CHAPTER SEVEN

IDENTIFICATION OF *ARMILLARIA* ISOLATES FROM BHUTAN BASED ON DNA SEQUENCE COMPARISONS

Accepted: *Plant Pathology* (2003)

IDENTIFICATION OF *ARMILLARIA* ISOLATES FROM BHUTAN BASED ON DNA SEQUENCE COMPARISONS

ABSTRACT

Armillaria root rot is a serious disease in fir and mixed conifer forests of Bhutan, Eastern Himalayas. The species causing this disease have, however, never been identified. The aim of this study was to identify field isolates collected at four localities in Bhutan. Identification was based on RFLP analysis of the IGS-1 region, comparisons of ITS and IGS-1 sequence data with those available on GenBank, cladistic analyses and sexual compatibility studies. Isolates were found to reside in two distinct RFLP groups. RFLP GROUP 1 isolates from *Pinus wallichiana* at Yusipang had RFLP profiles and IGS-1 sequences similar to those of *A. mellea* subsp. *nipponica*. Although ITS sequence data are not available for *A. mellea* subsp. *nipponica*, sequences from this DNA region were most similar to the closely related *A. mellea* from Asia. The RFLP profile and IGS-1 sequences for RFLP GROUP 2 isolates from *Abies densa* at Changaphug, *Tsuga dumosa* at Chimithanka as well as *Picea spinulosa* and *T. dumosa* in the Phobjikha valley were similar to those published for *A. borealis, A. cepistipes, A. gemina* and *A. ostoyae*. Parsimony analysis based on IGS-1 and ITS sequence data placed these isolates in a clade that included *A. calvescens, A. cepistipes, A. gallica, A. jezoensis, A. sinapina* and *A. singula*. The isolates were, however, sexually incompatible with tester strains of *A. calvescens, A. cepistipes, A. gallica* and *A. sinapina*. Although closely related to these species they appear to represent a distinct taxon that we will refer to as Bhutanese Phylogenetic Species I (BPS I) until basidiocarps are found and the species can be described.

Keywords: Armillaria root rot, *Armillaria mellea*, RFLP, IGS, ITS, biological species, phylogenetic species, Himalayas, Bhutan.

INTRODUCTION

Armillaria root rot is caused by various species of *Armillaria* (Tricholomataceae, Agaricales, Basidiomycetes). These fungi are pathogens occurring throughout temperate and most tropical regions of the world (Hood *et al.* 1991). *Armillaria* spp. survive as pathogens, saprobes or perthotrophs on woody trees and shrubs and tend not to show species-specific interactions with their hosts (Gregory *et al.* 1991, Termorshuizen 2001). These survival strategies make *Armillaria* spp. serious pathogens capable of inflicting severe losses in forests and plantations.

Historically, plant pathologists attributed Armillaria root rot to the single species *A. mellea,* based on the assumption that this is a highly pleomorphic species (Singer 1956). This view changed with the emergence of a biological species concept for *Armillaria* and the subsequent identification of new biological species in Europe and North America (Korhonen 1978, Ullrich and Anderson 1978, Anderson and Ullrich 1979). Based on morphological differences and sexual compatibility interactions, at least 36 species are now accepted in *Armillaria* (Volk and Burdsall 1995).

A contemporary approach to the identification of *Armillaria* spp. has been to use DNA-based characteristics. Consequently, restriction fragment length polymorphism (RFLP) profiles (Harrington and Wingfield 1995) and DNA sequence data from the internal transcribed spacer (ITS) (Coetzee *et al.* 2000, 2001) as well as the intergenic spacer region one (IGS-1) (Anderson and Stasovski 1992) of the rRNA operon, have become available for most commonly-known *Armillaria* spp. This has facilitated rapid identification of field isolates for which basidiocarps are not available.

The Kingdom of Bhutan is a small land-locked country, located in the Eastern Himalayas between China and India. The total area is 47 010 km^2 with 64.2% covered by forest (FAO 2001). The dense forest cover of Bhutan is exceptional for Southern and South-Eastern Asia that has generally been severely deforested. Forests are of immense socio-economic and ecological importance to Bhutan. Diseases affecting this natural resource, therefore, pose a great threat to the economic and social well-being of the country.

Very little is known regarding diseases in Bhutanese forests. Recent surveys have recorded a number of diseases of which Armillaria root rot was commonly encountered (Donaubauer 1986, 1993, Nedomlel 1994, Tshering and Chhetri 2000, Kirisits *et al.* 2002). Based on basidiocarp morphology Nedomlel (1994) recorded the presence of *A. ostoyae* in Bhutan. Apart from this record, virtually nothing is known regarding the identity of the *Armillaria* spp. causing root rot in conifer forests of this Himalayan country.

During the course of a survey of tree diseases in 2001 (Kirisits *et al.* 2002), typical symptoms and signs of Armillaria root rot were found in various conifer forests in Bhutan. These symptoms and signs included trees dying in patches and white mycelial mats below the bark, at the bases of dead and dying trees (Morrison *et al.* 1991). Rhizomorphs were also present in the soil and under the bark of dead and dying trees. Although basidiocarps were never encountered, it was possible to obtain diploid *Armillaria* isolates from dying trees. The aim of this study was, therefore, to identify field isolates from Bhutan using RFLP and DNA sequence data. In addition, results from these DNA based studies were evaluated using sexual compatibility tests with appropriate haploid tester strains.

MATERIALS AND METHODS

Collection sites

A total of thirteen *Armillaria* isolates were collected from trees in fir and mixed conifer forests at four locations in Bhutan, during July of 2001 (Table 1). Collection sites included Changaphug, Yusipang and Chimithankha in the Western part of the country and the Phobjikha valley in Central Bhutan (Fig. 1). The high altitude forests at Changaphug that consist of Eastern Himalayan fir (*Abies densa*), suffered severely from a disease syndrome, known as fir decline (Donaubauer 1993), in the 1980's, which resulted in the death of the majority of the trees at this site. This dramatic and wide-spread decline of fir in Western Bhutan was thought to be primarily caused by prolonged drought, but various biotic agents, including *Armillaria* spp., were suggested to be involved as contributing factors in the syndrome (Donaubauer 1986, 1987, 1993, Ciesla and Donaubauer 1994). In the Phobjikha valley, isolates were collected in a stand of Eastern Himalayan spruce (*Picea spinulosa*), suffering from a local outbreak of the bark beetle *Ips schmutzenhoferi* (Schmutzenhofer 1988, Kirisits *et al.* 2002). Obvious signs of Armillaria root rot were present on spruce trees, attacked by *I. schmutzenhoferi*. In addition to spruce, one

isolate was collected from Himalayan hemlock (*Tsuga dumosa*) in the Phobjikha valley. At Yusipang and Chimithankha, isolates were collected from Himalayan blue pine (*Pinus wallichiana*) and Himalayan hemlock, respectively. Armillaria root rot was not obvious on living trees at the latter sites but the isolates were included to gain a broader view of the occurrence and species composition of *Armillaria* spp. in Bhutan.

Fungal isolation and cultivation

Isolates were obtained either from mycelial fans or from rhizomorphs found between the bark and the wood of dying trees or on stumps. Small samples from the mycelial fans were placed on selective DBS (Dichloran-Benomyl-Streptomycin) medium (Harrington *et al.* 1992) and incubated at about 20 °C in artificial light for 2 weeks. Rhizomorphs from infected trees or stumps were surface sterilized in 96% ethanol for 1 min; small pieces from the inner parts were excised and placed on MA (2% Malt extract and 1.6% Agar) or selective DBS medium. Mycelium or rhizomorph tips, growing from primary isolates, were transferred to fresh medium and incubated. This procedure was repeated until pure cultures were obtained. Pure cultures were maintained on MYA (1.5% Malt extract, 0.2% Yeast extract and 1.5% Agar) medium. All isolates obtained from Bhutan are maintained in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI) (CMW), University of Pretoria, Pretoria, South Africa and the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Vienna, Austria.

DNA extraction

Armillaria isolates were grown in liquid MY (1% Malt and 2% Yeast extract) medium at 24 °C for four weeks in the dark. Mycelium was harvested using a sterile metal strainer, frozen at –70 °C for 20 min and lyophilized. The freeze-dried mycelium was then ground to a fine powder in liquid nitrogen. DNA extraction from the powdered mycelium followed the method described by Coetzee *et al.* (2000).

Amplification of the ITS and IGS-1 regions

The ITS region (ITS1, 5.8S and ITS2) of the rRNA operon was amplified using primer set ITS1/ITS4 (White *et al.* 1990). The IGS-1 region was amplified with primers CLR12R

(Veldman *et al.* 1981) and O-1 (Duchesne and Anderson 1990). The PCR mixture and conditions for amplification of the ITS and IGS-1 regions were the same as those described by Coetzee *et al.* (2000). Amplified ITS and IGS-1 PCR products were visualized on an agarose gel (1% agar) stained with ethidium bromide under UV illumination.

RFLP analysis of the IGS-1

Restriction enzyme digestion was done after PCR reactions by adding 10 U of the endo-nuclease *Alu*I to unpurified PCR mix (20 µL) containing the IGS-1 amplicons. DNA fragments were separated on an agarose gel (3%) stained with ethidium bromide and visualized under UV illumination. RFLP fragment sizes larger than 100 bp. were determined with GelFrag version 2.0.5 (National Centre for Super Computing Applications, University of Illinois at Urbana Champaign). RFLP profiles obtained for the isolates were compared with those previously published for various *Armillaria* spp. from Asia, Europe and North America (Harrington and Wingfield 1995, Schulze *et al.* 1995, Banik *et al.* 1996, Volk *et al.* 1996, Coetzee 1997, Chillali *et al.* 1998, Frontz *et al.* 1998, Terashima *et al.* 1998, White *et al.* 1998, Pérez Sierra *et al.* 1999, Coetzee *et al.* 2000, Kim *et al.* 2000, 2001).

DNA sequencing

DNA sequences for the ITS and IGS-1 regions were obtained using an ABI PRISM™ automated sequencer. PCR products were purified from unincorporated nucleotides and primer dimers prior to sequencing using a QIAquick PCR purification kit (QIAGEN, Germany) and eluted with 50 µL water. Sequence reactions were carried out with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, UK) following the protocol supplied by the manufacturer. The ITS region was sequenced in both directions using primers ITS1 and ITS4 as well as internal primers CS2B and CS3B (Coetzee *et al.* 2001). DNA sequences for the IGS-1 region were determined with primers P-1 (Hsiau 1996), O-1 and primers MCO2 and MCO2R (Coetzee *et al.* 2000) that anneal to a region in the middle of the IGS-1 region.

Cloning of IGS-1 amplicons

IGS-1 PCR products from isolates that gave poor sequencing results were cloned into vector pCR®4-TOPO® after purification, as outlined above. Cloning reactions were done using a TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Carlsbad, California) with One Shot® TOP10 Chemically Competent *E. coli* cells following the manufacturer's directions. Positive inserts were verified by amplifying the IGS-1 directly from transformed *E. coli* cells. The PCR mixture included dNTPs (250 µM each), *Taq* Polymerase (2.5 U) (Roche Diagnostics, Mannheim, Germany), PCR buffer with MgCl2 (supplied by the manufacturer) and primers P-1 and O-1 (0.1 µM each). The final volume of the PCR reaction mix was brought to 50 µL with water. PCR conditions were as follows: 1 cycle at 95 °C for 1 min (denaturation), 35 cycles of 60 °C for 30 s (primer annealing), 70 °C for 30 s (elongation) and 95 °C for 30 s (denaturation). A final elongation step was allowed at 70 °C for 7 min. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide. Two to three IGS-1 PCR products that had been successfully amplified from positively transformed cells were sequenced as described above.

Sequence and phylogenetic analyses

The identity of Bhutanese isolates was further investigated by comparing the ITS and IGS-1 sequences from representative isolates with sequence data available on the NCBI (National Center for Biotechnology Information) databases using a nucleotide BLAST (Basic Local Alignment Search Tool) search. This was followed by phylogenetic analyses to determine the relationship between the Bhutanese isolates and the *Armillaria* species, with which they had a high sequence similarity. ITS and IGS-1 DNA sequences for representative isolates from Bhutan were aligned with sequences of various *Armillaria* spp. available on GenBank. Alignment was done with Clustal X (Thompson *et al.* 1997) and manually corrected. Phylogenetic analysis was based on parsimony methods using PAUP* version 4.10 (Swofford 1998). Indels larger than two base pairs were coded using a multistate character system as outlined by Coetzee *et al.* (2001). Missing, parsimony-uninformative and ambiguously aligned regions were excluded from the data sets before analyses. Gaps were treated as a fifth character, "newstate". Most parsimonious trees were generated by heuristic searches with random addition of sequences (100 replicates), TBR (tree bisection reconnection) branch swapping and MULPARS active. MaxTrees was set to auto-increase after 100 MP trees were generated and

branches collapsed if negative branch lengths were obtained. Most parsimonious trees obtained were optimized by applying successive weighting of parsimony-informative characters according to their mean consistency index. Confidence in branching points was determined by bootstrap analysis (1000 replicates) (Felsenstein 1985).

Sexual compatibility tests

Diploid isolates belonging to RFLP GROUP 2, were paired with haploid tester strains of *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. gemina*, *A. mellea* and *A. sinapina* (Table 2) to confirm the results from DNA-based identifications. Sexual compatibility tests were conducted on MEA (1.5% Difco malt extract, 1.5% Difco agar) medium. Small (2 mm diam) plugs from diploid Bhutanese cultures and haploid tester strains were placed 5 mm apart on the medium and incubated at 24 °C in the dark. Mating reactions were evaluated after 4 and again after 6 weeks. Sexual compatibility tests were conducted at both FABI (all tester strains) and IFFF (only for *A. cepistipes* and *A. gallica*).

RESULTS

RFLP analysis

All isolates from Bhutan resided in one of two groups based on their RFLP profiles (Fig. 2). These are, hereafter, referred to as RFLP GROUP 1 and RFLP GROUP 2 isolates. RFLP GROUP 1 isolates had a profile with fragment sizes of 376 (374-379) and 166 (165-167) bp. This profile corresponded most closely to that of *A. mellea* subsp. *nipponica* from Japan (Terashima *et al.* 1998).

The fragment sizes for isolates in RFLP GROUP 2 were 309 (305-316), 195 (189-199) and 139 (137-141) bp. Some variation was, however, observed amongst banding patterns for these isolates. Isolate CMW10578 (Phob6), from the Phobjikha valley, had a profile slightly different to those of the other isolates. RFLP fragment sizes for this isolate were 417, 313, 198 and 138 bp. A species name could not be assigned to isolates residing in RFLP GROUP 2 because the banding patters were similar to those of *A. borealis*, *A. cepistipes*, *A. gemina* and *A. ostoyae* (Harrington and Wingfield 1995, Pérez Sierra *et al.* 1999, Kim *et al.* 2001).

Sequence analyses

RFLP GROUP 1 isolates

IGS-1 DNA sequences for isolates CMW8082 and CMW8202 from Yusipang residing in RFLP GROUP 1, were most similar to those of *A. mellea* from Japan (AF163610) and South Korea (AF163613, AF163612 and AF163611) and *A. mellea* subsp. *nipponica* (D89922) (98%). The highest blast score (932 bits) was obtained with *A. mellea* (AF163610) from Japan. The highest ITS sequence identity for these Bhutanese isolates was with *A. mellea* (98%) from South Korea (AF163592, AF163593 and AF163591).

Phylogenetic trees generated from IGS-1 sequences (Fig. 3) placed isolates CMW8082 and CMW8202 in a strongly supported monophyletic group that included *A. mellea* s. str. from Japan (AF163610) and South Korea (AF163611, AF162613) as well as *A. mellea* subsp. *nipponica* (100% bootstrap support). Most parsimonious trees obtained from ITS sequences (Fig. 4) placed the two isolates in a strongly supported monophyletic group (100% bootstrap support) that included isolates representing *A. mellea* s. str. from Japan (AF163594) and South Korea (AF163592 and AF163593).

RFLP GROUP 2 isolates

The IGS-1 amplicons for representative isolates residing in RFLP GROUP 2 could not be sequenced directly and the fragments were subsequently cloned. Sequence heterogeneity within the IGS-1 repeat region of the rDNA was observed when comparing cloned IGS-1 amplicons from the same individual. IGS-1 sequence comparisons indicated the presence of one 4 bp. indel and eleven nucleotide substitution sites with five of these sites being unique to CMW10578 (Fig. 5).

The highest IGS-1 sequence similarity for isolate CMW10583 from the Phobjikha valley, was with *A. cepistipes* (AF243069 and D89919), *A. sinapina* (D89925), *A. jezoensis* (D89921) and NABS X (AF243062). Although IGS-1 sequences of these species were all 97% similar to those of the isolate from Bhutan, the highest blast score was obtained with *A. cepistipes* and NABS X (888 bits). ITS sequences for isolate CMW10583 had the highest identity with ITS sequences for *A. cepistipes* (AJ250053) (99%, 1501 bits).

Parsimony trees generated from the IGS-1 region grouped representative isolates (CMW8095, CMW10578, CMW10581 and CMW10583) from RFLP GROUP 2 in a strongly supported clade (Fig. 6). Isolate CMW10578 from Phobjika valley, which had a different RFLP pattern, grouped within this clade with a 95% bootstrap support. RFLP GROUP 2 isolates formed a sister group to *A. cepistipes* (D89919), *A. sinapina* (D89925), *A. jezoensis* (D89921) and *A. singula* (D89926) from Japan, but this relationship had only a 50% bootstrap support. Most parsimonious trees generated from the ITS data set placed isolates CMW10583, CMW10581, CMW8095 and CMW8096 from Bhutan in a clade that included *A. cepistipes* (U54811, U54810 and AJ250053) and *A. gallica* (U54814, U54814 and AJ250054) with a 55% bootstrap support (Fig. 4). *Armillaria sinapina* (AF169646) formed a sister taxon to this clade with a 74% bootstrap support.

Sexual compatibility tests

Haploid tester strains representing *A. calvescens, A. cepistipes, A. gallica*, and *A. sinapina* were used for sexual compatibility tests because of their phylogenetic relationships with RFLP GROUP 2 isolates. Tester strains of *A. mellea* and *A. gemina*, two species distantly related to the Bhutanese isolates, were included as negative controls. The haploid tester strains of these species retained their fluffy, white aerial mycelium when crossed with diploid isolates in RFLP GROUP 2 (Fig. 7). Demarcation lines were also observed where mycelial growth from the different inocula interacted. These results indicate that the RFLP GROUP 2 isolates from Bhutan are sexually incompatible with the tester strains included in this study.

DISCUSSION

This study represents a first attempt to identify a reasonably large collection of *Armillaria* isolates from Bhutan. The isolates were from a variety of locations and hosts at different altitudes in Bhutan and we, therefore, anticipated finding a variety of *Armillaria* spp. RFLP analyses, however, showed that all isolates resided in one of two distinct groups that could easily be recognised.

RFLP profiles of Bhutanese isolates from *P. wallichiana* at Yusipang (RFLP GROUP 1) were similar to those previously published by Terashima *et al.* (1998) for the homothallic *A. mellea*

subsp. *nipponica* from Japan. It was, therefore, suspected that RFLP GROUP 1 isolates from Bhutan represent this subspecies of *A. mellea*. Phylogenetic analyses based on parsimony that incorporated IGS-1 and ITS sequence data were subsequently used to confirm this finding. Parsimony trees generated in this study grouped the RFLP GROUP 1 isolates in a strongly supported monophyletic Asian *A. mellea* subclade, comprised of isolates from Japan and Korea. This subclade included *A. mellea* subsp. *nipponica* in cladograms generated from IGS-1 sequence data. The strongly supported grouping of this subspecies of *A. mellea* within the Asian subclade suggests that other isolates included in this clade also represent *A. mellea* subsp. *nipponica*. Based on these findings we believe that the Bhutanese RFLP GROUP 1 isolates belong to *A. mellea* subsp. *nipponica*.

Direct sequencing of the IGS-1 PCR products for representative isolates residing in RFLP GROUP 2 was difficult, despite various attempts using different reaction conditions. The IGS-1 region forms part of the tandemly repeated rDNA multigene family (Long and Dawid 1980). Sequences from a limited number of cloned IGS-1 fragments showed sequence heterogeneity among multi-copies of this region; indicating intragenomic IGS-1 sequence variation within individuals. Our limited data further indicated that the IGS-1 could be separated into two non-orthologous (homologs that are not the result of speciation) intragenomic types based on the presence or absence of a four base pair indel.

It was not possible to fully resolve the identity of isolates residing in RFLP GROUP 2. This was firstly because their RFLP profiles resembled those of more than one *Armillaria* sp. Furthermore, there was poor statistical support for groupings based on phylogenetic analyses of ITS and IGS-1 sequences. However, it was clear that RFLP GROUP2 isolates are closely related to *A. cepistipes*, *A. sinapina* and *A. gallica*. The isolates are, therefore, considered to be part of the "*A. gallica* cluster" that includes *A. cepistipes*, *A. gallica*, *A. sinapina* and various other *Armillaria* spp. from the Northern Hemisphere (Korhonen 1995). Species residing in this group are similar in having basidiocarps with a delicate annulus and a bulbouse stipe-base, thin cylindrical monopodially branching rhizomorphs, and saprophytic or weakly parasitic life cycles (Korhonen 1995).

Isolates from Chimitankha, Changaphug and all but one of the isolates from Phobjika valley had the same RFLP profiles and most likely represent a single taxon. Isolate CMW10578 from *P. spinulosa* in Phobjika valley was, however, the exception in having a RFLP profile slightly

different to the rest of the RFLP GROUP 2 isolates. IGS-1 sequence data obtained for this isolate showed that a number of unique base substitutions were present, thus explaining the anomalous RFLP results. Phylogenetic analyses, however, placed this isolate in a strongly supported clade that included representative isolates from the same region and host. Despite RFLP and IGS-1 sequence variation, this isolate (CMW10578) is, therefore, thought to represent the same species as others in RFLP GROUP 2.

Representative isolates in RFLP GROUP 2 could not be identified based on mating tests. These isolates were clearly intersterile with those species (*A. calvescens*, *A. cepistipes*, *A. gallica* and *A. sinapina*) phylogenetically most closely related to them. Isolates of RFLP GROUP 2, therefore, either represent an undescribed taxon or one of the Indian (Himalayan) *Armillaria* spp. (Chandra and Watling 1981) for which neither tester strains for matings, reference cultures, nor molecular data are available. Until basidiocarps linked to this group of isolates can be found and collected, their exact identity cannot be resolved. For the present, we will refer to them as Bhutanese Phylogenetic Species I (BPS I).

REFERENCES

Anderson JB, Ullrich RC. 1979. Biological species of *Armillaria mellea* in North America. *Mycologia* **71**: 402 - 414.

Anderson JB, Stasovski E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycologia* **84**: 505 - 516.

Banik MT, Volk TJ, Burdsall HH. 1996. *Armillaria* species of the Olympic Peninsula of Washington state, including confirmation of North America biological species IX. *Mycologia* **88**: 492 - 496.

Chandra A, Watling R. 1981. Studies in Indian *Armillaria* (Fries per Fries) Staude (Basidiomycotina). *Kavaka* **10**: 63 - 84.

Chillali M, Idder-Ighili H, Guillaumin J-J, Mohammed C, Lung Escarmant B, Botton B. 1998. Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European *Armillaria*. *Mycological Research* **102**: 533 - 540.

Ciesla W, Donaubauer E, 1994. *Decline and Dieback of Trees and Forests. A Global Overview*. Rome, Italy: Food and Agriculture Organization of the United Nations, FAO Forestry Paper 120.

Coetzee MPA. 1997. *Characterisation of* Armillaria *in South Africa.* Bloemfontein, South Africa: University of the Orange Free State, MSc thesis.

Coetzee MPA, Wingfield BD, Harrington TC, Dalevi D, Coutinho TA, Wingfield MJ. 2000. Geographical diversity of *Armillaria mellea* s.s. based on phylogenetic analysis. *Mycologia* **92**: 105 - 113.

Coetzee MPA, Wingfield BD, Bloomer P, Ridley GS, Kile GA, Wingfield MJ. 2001. Phylogenetic relationships of Australian and New Zealand *Armillaria* species. *Mycologia* **93**: 887 - 896.

Donaubauer E. 1986. *Technical Advisory Services for Forest Development, Bhutan, Forest Pathology.* FO/DP/BHU/83/022, Field Document 11. Thimphu, Bhutan: Department of Forests, Ministry of Trade, Industry and Forests; Rome, Italy, FAO.

Donaubauer E. 1987. *Technical Advisory Services for Forest Development, Bhutan, Forest Pathology.* FO/DP/BHU/83/022, Field Document 12. Thimphu, Bhutan: Department of Forests, Ministry of trade, Industry and Forests; Rome, Italy: FAO.

Donaubauer E. 1993. On the decline of fir (*Abies densa* Griff.) in Bhutan. In: Huettl RF, Mueller-Dombois, eds. *Forest Decline in the Atlantic and Pacific Region.* Berlin, Heidelberg, Germany: Springer-Verlag, 332 - 337.

Duchesne LC, Anderson JB. 1990. Location and direction of transcription of the 5S rRNA gene in *Armillaria*. *Mycological Research* **94**: 266 - 269.

FAO. 2001. *Global Forest Resources Assessment 2000 – Main report.* FAO Forestry Paper 140. Rome, Italy.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39**: 783 - 791.

Frontz TM, Davis DD, Bunyard BA, Royse DJ. 1998. Identification of *Armillaria* species isolates from bigtooth aspen based on rDNA RFLP analysis. *Canadian Journal of Forestry Research* **28**: 141 - 149.

Gregory SC, Rishbeth J, Shaw CG. 1991. Pathogenicity and virulence. In: Shaw CG, Kile GA, eds. *Armillaria Root Disease.* USDA Agriculture Handbook No. 691. Washington DC, USA: Forest Service, United States Department of Agriculture, 76 - 87.

Harrington TC, Worrall JJ, Baker FA. 1992. *Armillaria*. In: Singleton LL, Mihail JD, Rush C, eds. *Methods for Research on Soil-Borne Phytopathogenic Fungi.* St. Paul, USA: American Phytopathological Society Press, 81 - 85.

Harrington TC, Wingfield BD. 1995. A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**: 280 - 288.

Hood IA, Redfern DB, Kile GA. 1991. *Armillaria* in planted hosts. In: Shaw CG, Kile GA, eds. *Armillaria Root Disease.* USDA Agriculture Handbook No. 691. Washington,DC, USA: Forest Service, United States Department of Agriculture, 122 - 149.

Hsiau PT-W. 1996. *The taxonomy and Phylogeny of the Mycangial Fungi from* Dendroctonus brevicomis *and* D. frontalis *(Coleoptera: Scolytidae)* Ames, USA: Iowa State University, PhD thesis.

Kim M-S, Klopfenstein NB, McDonald GI, Arumuganthan K, Vidaver AK. 2000. Characterization of North American *Armillaria* species by nuclear DNA content and RFLP analysis. *Mycologia* **92**: 874 - 883.

Kim M-S, Klopfenstein NB, McDonald GI, Arumuganathan K, Vidaver AK. 2001. Use of flow cytometry, fluorescence microscopy, and PCR-based techniques to assess intraspecific and interspecific matings of *Armillaria* species. *Mycological Research* **105**: 153 - 163.

Kirisits T, Wingfield MJ, Chhetri DB. 2002. *Studies on the Association of Blue-Stain Fungi Associated with the Eastern Himalayan Spruce Bark Beetle (*Ips schmutzenhoferi*) and with Other Bark Beetles in Bhutan.* Yusipang Report YREP/2002/02.

Korhonen K. 1978. Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* **18**: 31 - 42.

Korhonen K. 1995. *Armillaria* since Elias Fries. *Acta Universitatis Upsaliensis Symbolae Botanicae Upsalienses* **30**: 153 - 161.

Long EO, Dawid IB. 1980. Repeated genes in eukaryotes. *Annual Review of Biochemistry* **49**: 727 - 764.

Morrison DJ, Williams RE, Whitney RD. 1991. Infection, disease development, diagnosis, and detection. In: Shaw CG, Kile GA, eds. *Armillaria Root Disease.* USDA Agriculture Handbook No. 691. Washington DC, USA: Forest Service, United States Department of Agriculture, 62 - 75.

Nedomlel C. 1994. *Forest Pathological Characterisation of* Abies densa *in the Integrated Forest Management Project Area.* Thimphu, Bhutan: Royal Government of Bhutan, Ministry of Agriculture, Department of Forestry; Vienna, Austria: ADC and FALCH Austria, Austrian association for development and cooperation.

Pérez Sierra A, Whitehead DS, Whitehead MP. 1999. Investigation of a PCR-based method for the routine identification of British *Armillaria* species. *Mycological Research* **103**: 1631 - 1636.

Schmutzenhofer H. 1988. Mass outbreak of *Ips* bark beetles in Bhutan and the revision of the genus *Ips* de Geer for the Himalayan region. In: Payne TL, Saarenmaa H, eds.

Proceedings of the IUFRO Working Party VII, International Congress of Entomology Symposium "Integrated control of Scolytid bark beetles". Vancouver BC, Canada, 345 - 355.

Schulze S, Bahnweg G, Tesche M, Sandermann H. 1995. Identification of European *Armillaria* species by restriction-fragment-length polymorphisms of ribosomal DNA. *European Journal of Forest Pathology* **25**: 214 - 223.

Singer R. 1956. The *Armillariella mellea* group. *Lloydia* **19**: 176 - 178.

Swofford DL. 1998. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Vers. 4. Sunderland, USA: Sinauer Associates.

Terashima K, Kawashima Y, Cha JY, Miura K. 1998. Identification of *Armillaria* species from Hokkaido by analysis of the intergenic spacer (IGS) regions of ribosomal DNA using PCR-RFLP. *Mycoscience* **39**: 179 - 183.

Termorshuizen AJ. 2001. Ecology and epidemiology of *Armillaria*. In: Fox RTV, ed. *Armillaria Root Rot: Biology and Control of Honey Fungus*. Andover, UK: Intercept Limited, 45 - 63.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876 - 4882.

Tshering G, Chhetri DB. 2000. *Important Forest Insect Pests and Diseases of Bhutan with Control Measures*. Field Guide 2000/1. Thimphu, Bhutan: Department of Research and Development Services, Ministry of Agriculture.

Ullrich RC, Anderson JB. 1978. Sex and diploidy in *Armillaria mellea*. *Experimental Mycology* **2**: 119 - 129.

Veldman GM, Klootwijk J, de Regt VCHF, Planta RJ, Branlant C, Krol A, Ebel J-P. 1981. The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Research* **9**: 6935 - 6952.

Volk TJ, Burdsall HH. 1995. *A Nomenclatural Study of* Armillaria *and* Armillariella *Species (Basidiomycotina, Tricholomataceae)*. Førde, Norway: Fungiflora.

Volk TJ, Burdsall HH, Banik MT. 1996. *Armillaria nabsnona*, a new species from western North America. *Mycologia* **88**: 484 - 491.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego, USA: Academic Press, Inc, 315 - 322.

White EE, Dubetz CP, Cruickshank MG, Morrison DJ. 1998. DNA diagnostic for *Armillaria* species in British Columbia: within and between species variation in the IGS-1 and IGS-2 regions. *Mycologia* **90**: 125 - 131.

TABLE 1: *Armillaria* isolates from Bhutan included in this study.

Isolate number	Alternative number	Location in Bhutan	Host tree
CMW8081	Yus1	Yusipang	*Pinus wallichiana*
CMW8082	Yus2	"	"
CMW8084	Yus3	"	"
CMW8202	Yus4	"	"
CMW8095	Cha1	Changaphug	*Abies densa*
CMW8096	Cha2	"	"
CMW10583	Phob2	Phobjikha valley	*Tsuga dumosa*
CMW10576	Phob3	"	*Picea spinulosa*
CMW10577	Phob4	"	"
CMW10578	Phob6	"	"
CMW10579	Phob7	"	"
CMW10581	Phob9	"	"
CMW10582	Chim2	Chimithankha	*T. dumosa*

TABLE 2: *Armillaria* isolates used as testers in the sexual compatibility tests.

Species	Isolate number	Other numbers	Origin	Collector	Host
A. calvescens	CMW6893	PR-2, ss-2	USA	Banik MT	*Acer rubrum*
A. cepistipes	CMW6909	82-14-14	Canada	Morrison DJ	unknown
"	CMW6912	HHB-14868, ss-2	USA	Banik MT	*Alnus rubra*
"	CMW11262	IFFF 416, 92165	Finland	Korhonen K.	*Salix caprea*
"	CMW11263	IFFF 417, 93288	Poland	Zólciak A.	unknown
"	CMW11269	IFFF 441	Unknown	Unknown	unknown
A. gallica	CMW3169	B500, ATCC52114	USA	Anderson JB	unknown
"	CMW11272	IFFF 451	unknown	unknown	unknown
A. gemina	CMW3166	B735, AMP4B	USA	Worrall JJ	unknown
"	CMW3181	B485, ATCC52102	USA	Anderson JB	unknown
"	CMW6889	TJV 94-47, ss-2	USA	Banik MT	*Quercus velutina*
A. sinapina	CMW6894	HHB-14911, ss-9	USA	Banik MT	*Tsuga heterophylla*
A. mellea	CMW6901	IL-7, ss-3	USA	Banik MT	*Ulmus* sp.
"	CMW11271	IFFF 448	Unknown	unknown	unknown

Figure 1. Map of Bhutan, showing the four collection sites.

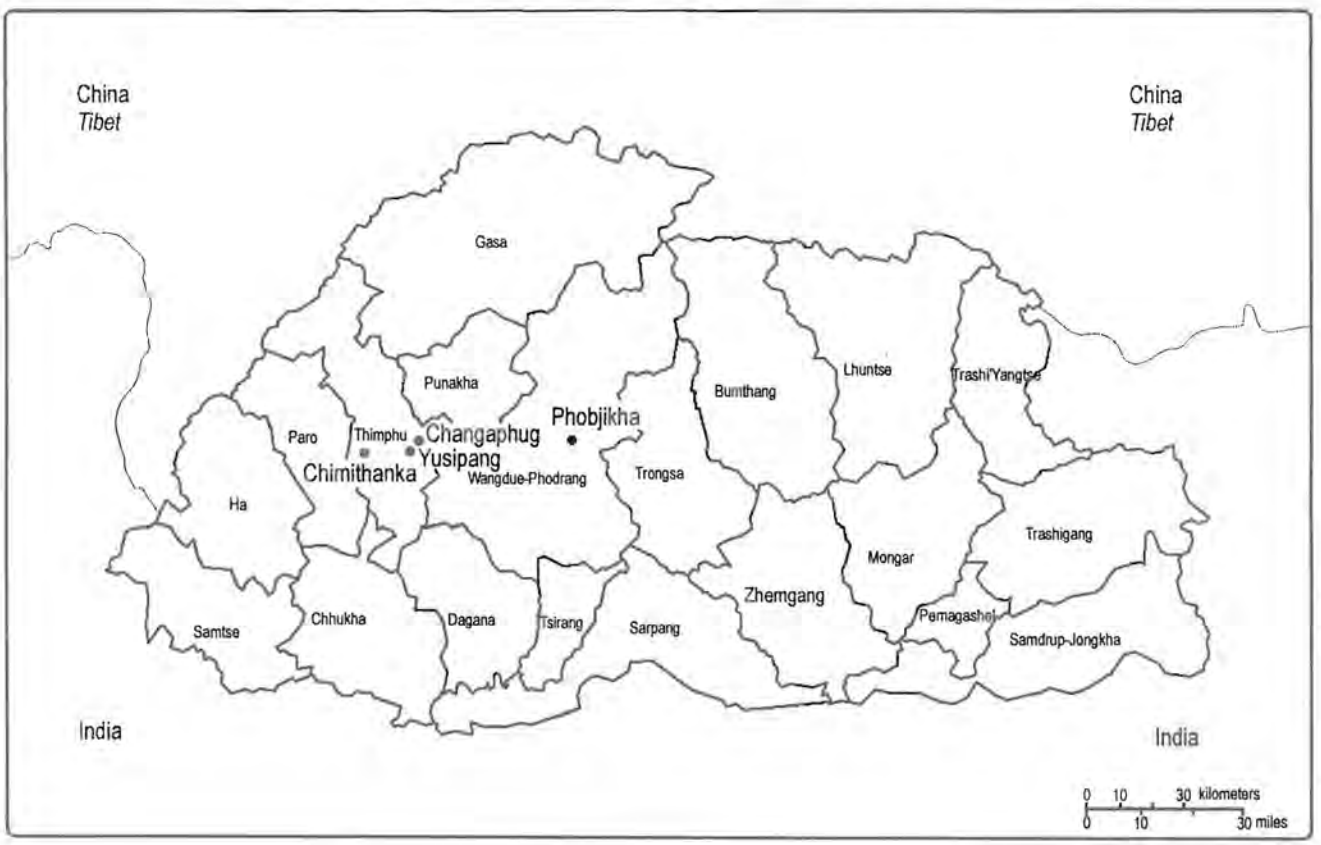

China
Tibet
China
Tibet
Gasa
Punakha
Phobjikha
Bumthang
Lhuntse
Trashi Yangtse
Paro
Thimphu
Changaphug
Yusipang
Chimithanka
Wangdue-Phodrang
Trongsa
Ha
Trashigang
Mongar
Zhemgang
Pemagashel
Samdrup-Jongkha
Samtse
Chhukha
Dagana
Tsirang
Sarpang
India
India
0 10 30 kilometers
0 10 30 miles

Figure 2. A 3% agarose gel stained with ethidium bromide showing *Alu*I restriction fragments for isolates of *Armillaria* from Bhutan. Lanes labeled M show a 100 bp. molecular marker (band sizes indicated in base pairs).

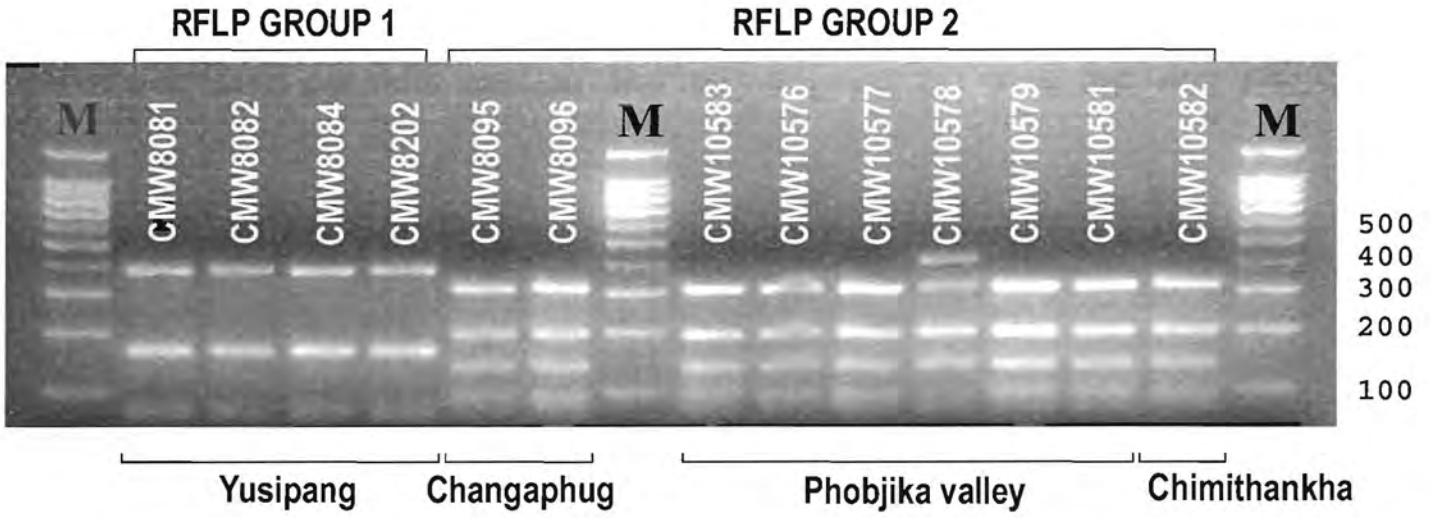


Figure 3. The single most parsimonious trees generated after a heuristic search in PAUP* using IGS-1 sequence data (782 characters, 180 parsimony informative characters) from RFLP GROUP 1. Tree length = 203 steps, *CI* = 0.929 and *RI* =0.958. Numbers above and below the tree branches indicate the branch length and the bootstrap support values for the branching nodes, respectively. The tree is rooted to *A. ostoyae*.

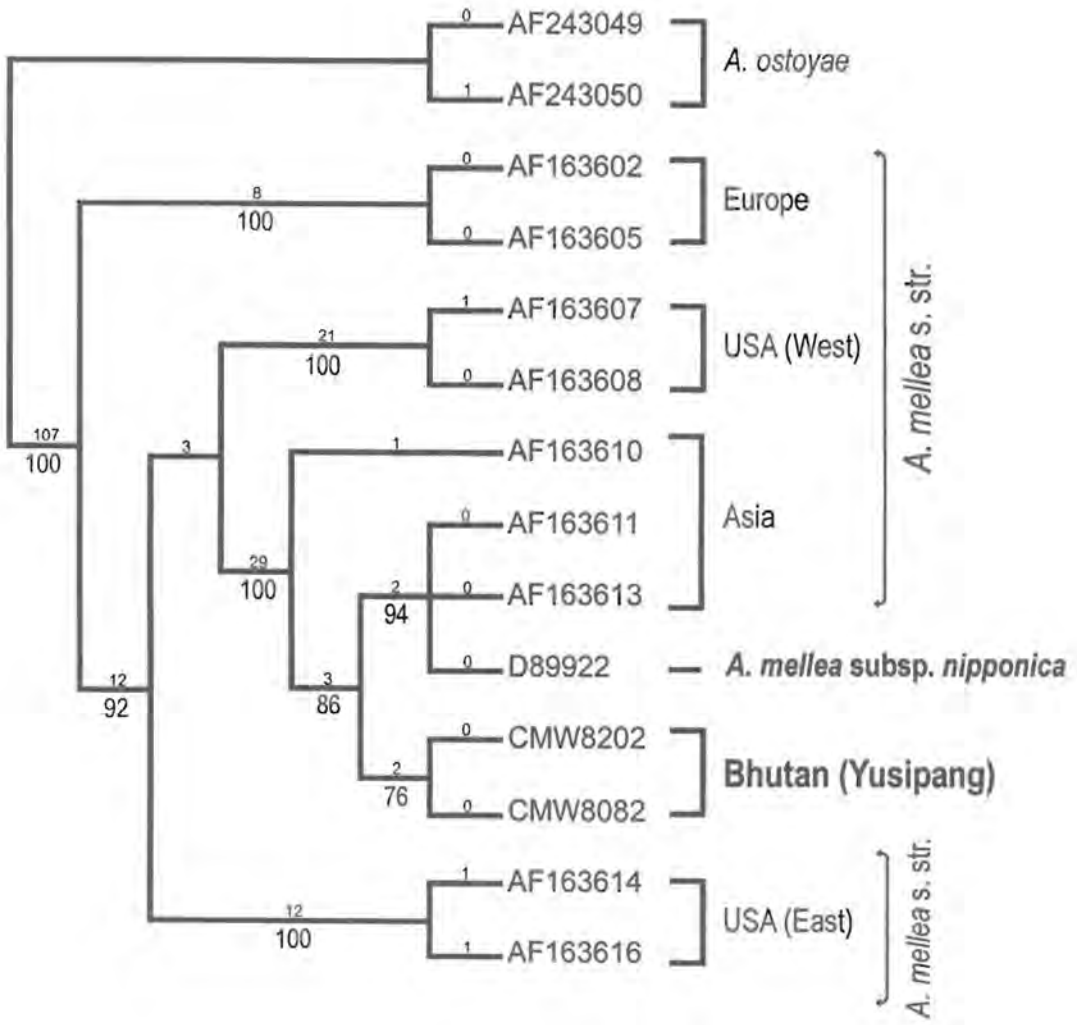

AF243049
AF243050
A. ostoyae
AF163602
AF163605
Europe
AF163607
AF163608
USA (West)
AF163610
AF163611
AF163613
Asia
A. mellea s. str.
D89922
A. mellea subsp. nipponica
CMW8202
CMW8082
Bhutan (Yusipang)
AF163614
AF163616
USA (East)
A. mellea s. str.

Figure 4. One of 18 most parsimonious trees based on ITS sequence data (1033 characters, 266 parsimony informative characters) for RFLP GROUP 1 and 2 from Bhutan generated after a heuristic search in PAUP*. Tree length = 433 steps, *CI* = 0.849 and *RI* = 0.916. *Armillaria fuscipes* is used as outgroup.

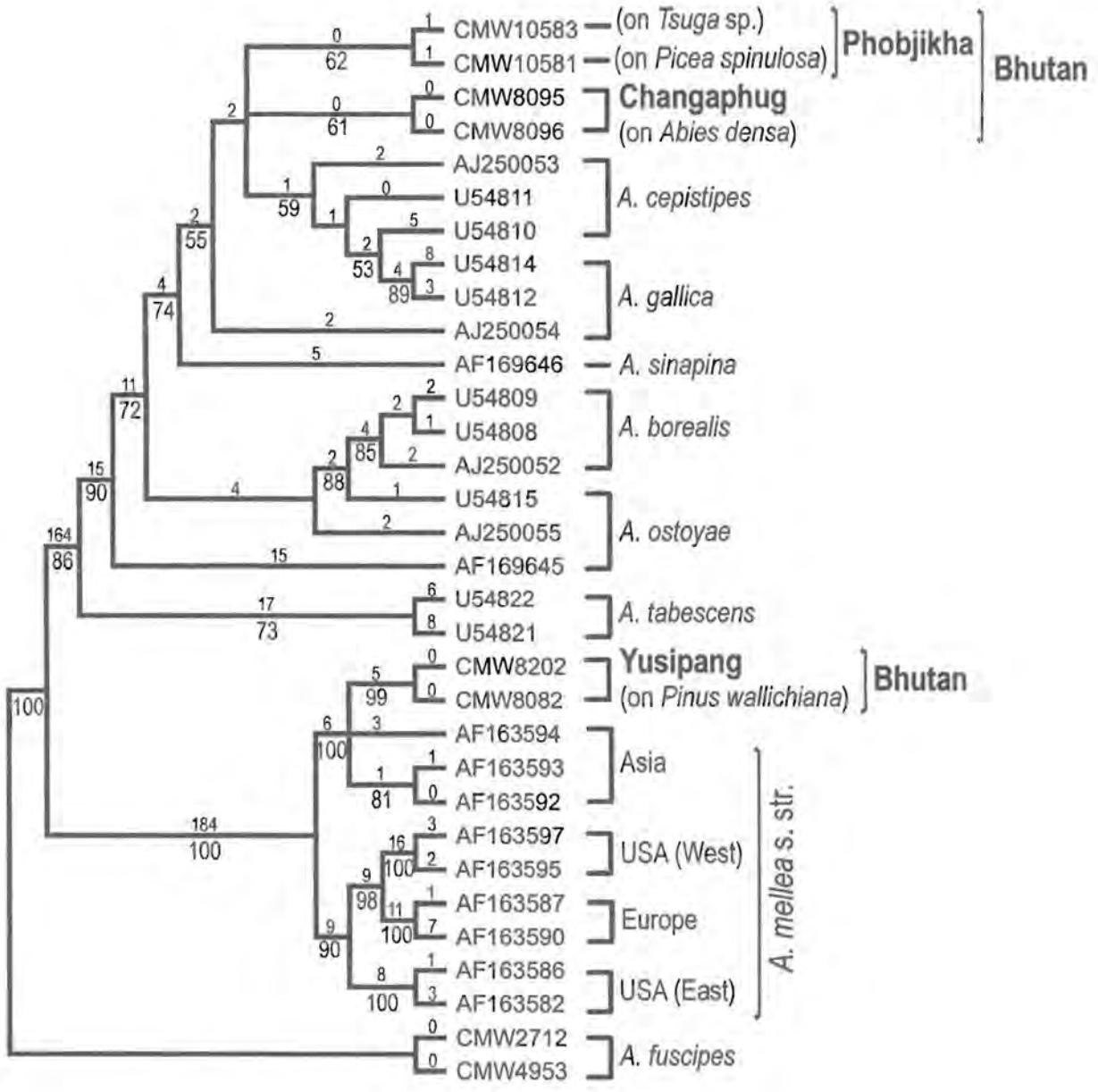


Figure 5. Alignment of DNA sequences from cloned IGS-1 fragments. Numbers (C) following the isolate number refer to the clone number. Dashes and stars below the sequences indicate homogeneous and heterogenous regions, respectively. Blocks in gray indicate sites with substitution unique for CMW10578 from Phobjikha valley that had a different RFLP pattern to the rest of the isolates from the same area as well as those from Chimithankha and Changaphug.

```
                    10        20        30        40        50        60
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10583c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10583c2          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c2          CGATCCGCTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10578c5          CGATCCACTGAGGTTAAGCTCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10581c1          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10581c2          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
CMW10582c4          CGATCCACTGAGGTTAAGCCCTTGTTCTAAAGATTTGTTCAACTTTGTTGGACTTTCTCT
                    ------------------*-----------------------------------------

                              70        80        90       100       110       120
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTTCTTTTTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10583c1          TTTCTTTTTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10583c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10578c1          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10578c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10578c5          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGAATAGTATCCTTTGTGCACTCGCGACA
CMW10581c1          TTTCTTTCTACATGCTGAGGCCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10581c2          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCCTTTGTGCACTCGCGACA
CMW10582c4          TTTCTTTCTACATGCTGAGACCTTGAGGGCCGGGATAGTATCcTTTGTGCACTCGCGACA
                    -------*---------------------------*---------------------------

                             130       140       150       160       170       180
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTT----A
CMW10583c1          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTT----A
CMW10583c2          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10578c1          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTCGTTA
CMW10578c2          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTTGTTA
CMW10578c5          GCATGTTACTTAGATTCGAAAGGGTAAGTTAACAACAATGCCTTAGTGTTTTGTTTGTTA
CMW10581c1          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10581c2          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
CMW10582c4          GCATGTTACTTAGATTCGAAAGGGTAAGCTAACAACAACGCCTTAGTGTTTTGTTTGTTA
                    ------------------------------*---------*-----------------****-

                             190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGGCTTATAAGGCACTTAGTTA
CMW10583c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10583c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGaAgACTTATAAGGCACTTAGTTA
CMW10578c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10578c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10578c5          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10581c1          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10581c2          CCTTTCTCCTTTGAATCACGAGTTATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
CMW10582c4          CCTTTCTCCTTTGAATCACGAGTCATTATGAGCCTTGAAGACTTATAAGGCACTTAGTTA
                    ------------------------------------------------------------
```

```
                    250        260        270        280        290        300
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10583c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGCTCTTTACCTTGTACTTGATATCGACTTT
CMW10583c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10578c5          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10581c1          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10581c2          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
CMW10582c4          GCAAGCTCTAACCGCGCGCTGACTTGGAACGGTCTTTACCTTGTACTTGATATCGACTTT
                    ------------------------------------------------------------

                    310        320        330        340        350        360
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10583c1          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10583c2          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c1          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c2          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10578c5          ATGGCCGATATCCCATATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10581c1          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10581c2          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
CMW10582c4          ATGGCCGATATCCCGTATATGGTATAGCCAAGATCCTTGAAAGGGCAAGTCAACGACTGA
                    ------------------------------------------------------------

                    370        380        390        400        410        420
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10583c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGAC
CMW10583c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10578c5          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10581c1          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10581c2          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
CMW10582c4          TTTTCTGGATCGTTAGTGAGCTTGAGGGTCTGCCCTAAGGTTGCCATGATTGAAAAGGCC
                    ------------------------------------------------------------

                    430        440        450        460        470        480
                    ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3           TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10583c1          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10583c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTCACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10578c1          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10578c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10578c5          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACCTTTTTAACCGTTTCAACCGTTTACTTAG
CMW10581c1          TTAGAAGCTAAGTAAGTTAAGCTATGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10581c2          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
CMW10582c4          TTAGAAGCTAAGTAAGTTAAGCTACGGTTACTTTTTTAACCGTTTCAACTGTTTACTTAG
                    -------------------------*---*--*-----------------*----------
```

```
                490       500       510       520       530       540
           ....|....|....|....|....|....|....|....|....|....|....|....|
CMW8095c3  CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10583c1 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10583c2 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10578c1 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10578c2 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10578c5 CTTTCGAGGGCTAGGTTCAAAATTTGAACGGCAACTTGTTCTGAAACGAAAGGTTTGCTA
CMW10581c1 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10581c2 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
CMW10582c4 CTTTCGAGGGCTACGTTCAAAATTTGAACGGCAACTGGTTCTGAAACGAAAGGTTTGCTA
           ----------*----------*----------

                550       560       570       580       590
           ....|....|....|....|....|....|....|....|....|....|....
CMW8095c3  AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10583c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10583c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10578c5 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10581c1 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10581c2 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
CMW10582c4 AGTAAACCATTGGTCAAGACCGGTTTGCAACAATTTTGGTGGCTGTAGGGTGAG
           ----------
```

Figure 6. One of five most parsimonious trees based on IGS-1 sequence data (531 characters, 125 parsimony informative characters) for isolates from RFLP GROUP 2 after a heuristic search in PAUP*. Tree length = 162 steps, *CI* = 0.878 and *RI* = 0.949. C-numbers indicate the clone number for a specific isolate. Numbers above and below the tree branches indicate the branch length and the bootstrap support values for the branching nodes, respectively. The outgroup taxon for this tree is *A. hinnulea*.

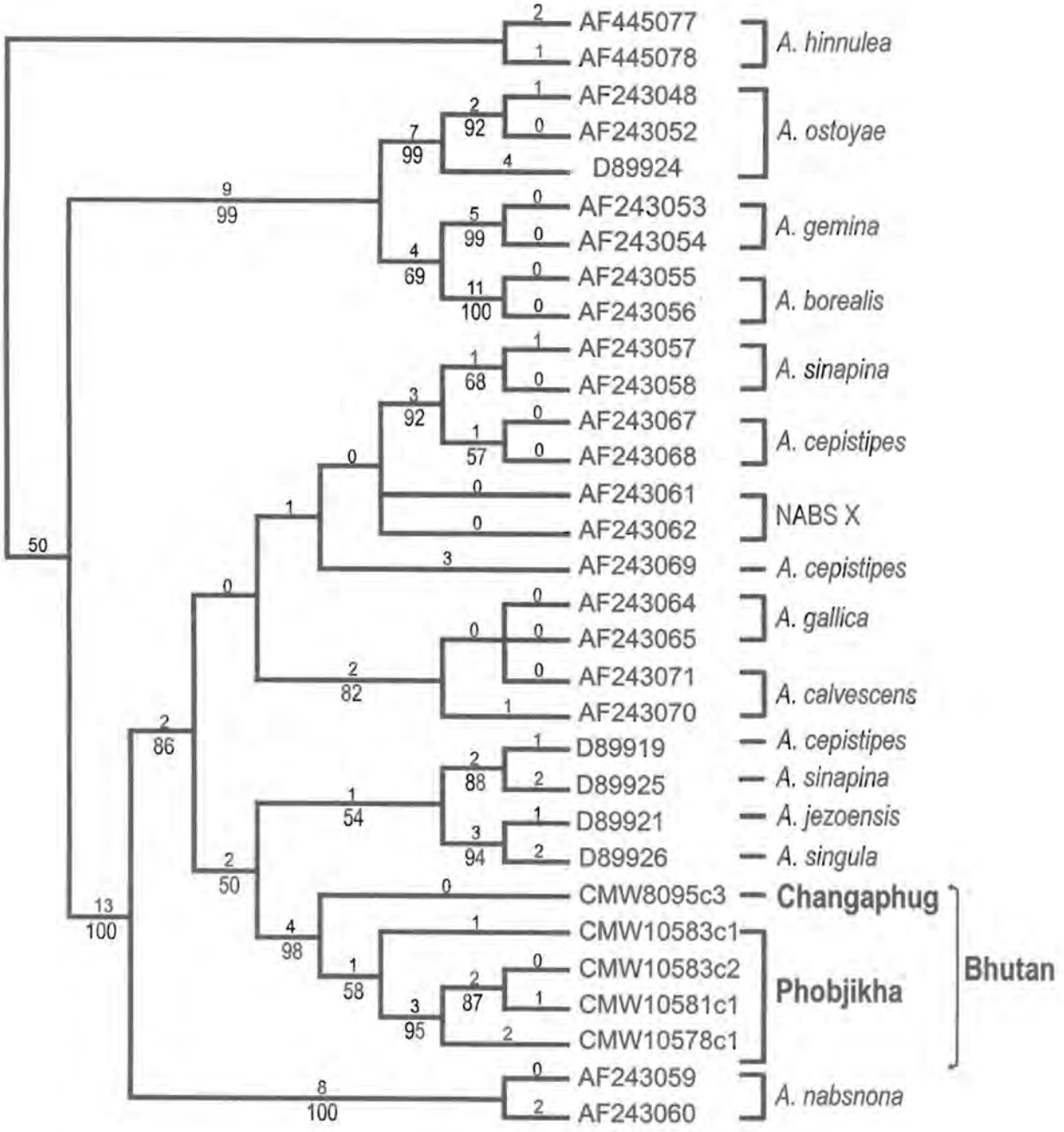
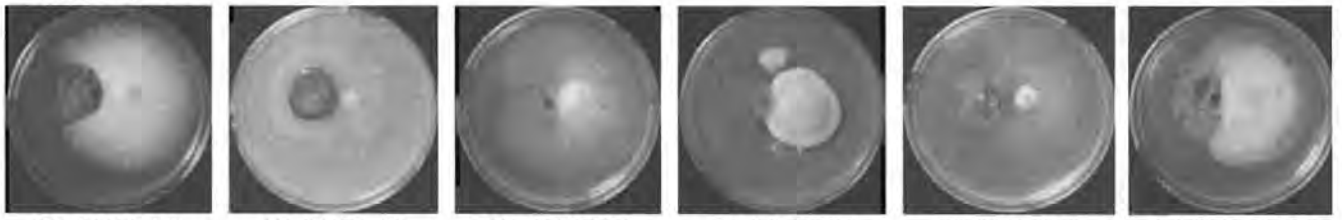


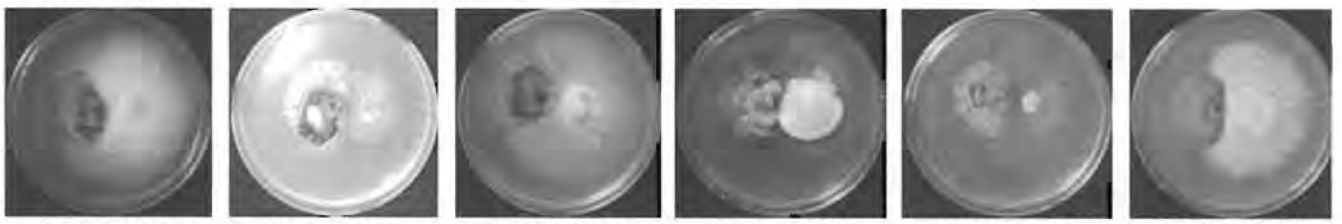
Figure 7. Two examples of results obtained after sexual compatibility tests between diploid RFLP GROUP 2 isolates from Bhutan (left inoculum) and haploid tester strains (right inoculum).

CMW9585



CMW6909 *A. cepistipes* | CMW3169 *A. gallica* | CMW6894 *A. sinapina* | CMW3181 *A. gemina* | CMW6893 *A. calvescens* | CMW11271 *A. mellea*

CMW9588



CMW6909 *A. cepistipes* | CMW3169 *A. gallica* | CMW6894 *A. sinapina* | CMW3181 *A. gemina* | CMW6893 *A. calvescens* | CMW6901 *A. mellea*

CHAPTER EIGHT

RFLP IDENTIFICATION TOOL FOR *ARMILLARIA* SPECIES

RFLP IDENTIFICATION TOOL FOR *ARMILLARIA* SPECIES

ABSTRACT

Armillaria spp. cause an important disease known as Armillaria root rot on woody plants throughout the world. Strategies to monitor and control this disease require correct and efficient identification of species. Identification of *Armillaria* spp. is typically based on basidiocarp morphology, which is complicated by the fact that these structures are rare and ephemeral. Sexual compatibility tests between isolates are also used for identification, but these are time consuming and often yield ambiguous results. Recently, restriction fragment length polymorphisms of the ITS and IGS-1 rRNDA regions have been employed and are now standard procedure for rapid and effective identification of *Armillaria* spp. The extensive use of this method has yielded a large number RFLP profiles for different species, which are available from a substantial and rapidly expanding suite of publications. Identification following this approach consequently requires a large number of comparisons between RFLP profiles of unknown isolates with those that have been published. This is a procedure that is becoming increasingly cumbersome. We have, therefore, developed an electronic database of published profiles and an automated search algorithm for rapid identification of *Armillaria* isolates. At present this application is "stand-alone" and includes RFLP profiles only from the ITS and IGS-1 rDNA regions of *Armillaria* spp. In future it will be converted to a WEB-based application and expanded to include profiles from other gene regions and genera.

Key words: RFLP, IGS, ITS, taxonomy.

INTRODUCTION

Armillaria (Basidiomycetes, Agaricales, Tricholomataceae) comprises a group of fungi causing the important disease known as Armillaria root rot. This disease is well known to plant pathologists due to the substantial losses that it can cause in natural forests, commercial forest plantations, horticultural crops and in agriculture where specifically cash crop plantations are damaged (Hood *et al.* 1991, Kile *et al.* 1991). The impact of Armillaria root rot is exacerbated by its cosmopolitan distribution (Hood *et al.* 1991). It thus poses a potential threat to industries based on woody crops and needs to be continually monitored and correctly managed.

Strategies for monitoring and managing Armillaria root rot disease require correct and efficient identification of the *Armillaria* spp. involved in the various disease syndromes. Historically, these fungi have been classified based on their basidiocarp morphology, but this poses several problems. These structures are seasonal and often unavailable when field surveys are conducted. They are also ephemeral and disappear within a relatively short period after sporulation. Furthermore, some of the *Armillaria* species have similar basidiocarp morphology and are difficult to distinguish from one another. It was largely due to these problems that the biological species concept was adopted for species recognition (Korhonen 1978, Anderson and Ullrich 1979, Guillaumin and Berthelay 1981). Identification based on recognition of biological species involves sexual compatibility tests between known haploid tester strains and cultures made from field samples. These tests are routinely employed in some laboratories but they are time consuming and often yield ambiguous results. In recent years, identification using DNA-based data has become increasingly common. This approach is relatively simple and time efficient. Thus, although a reasonable repertoire of methods is available to identify *Armillaria* spp., those based on DNA data are considered to be the most robust.

DNA-based data for *Armillaria* spp. identification are currently generated from DNA sequences and PCR-RFLPs (restriction fragment length polymorphisms) from the ITS and IGS-1 regions of the rRNA operon (e.g. Anderson and Stasovski 1992, Harrington and Wingfield 1995, Chillali *et al.* 1998, Coetzee *et al.* 2000b). Identification based on DNA sequences is hampered by the fact that generating and comparing sequence data is slow and expensive when large numbers of samples are to be processed. In contrast, PCR-RFLPs represent a relatively inexpensive and

rapid approach that does not require highly specialised services. These advantages lend impetus to the application of PCR-RFLP analysis as standard procedure for identifying *Armillaria* spp.

Extensive application of PCR-RFLPs by several research laboratories has yielded large numbers of RFLP profiles associated with various *Armillaria* spp. (Tables 1 and 2). These profiles are available from a large and rapidly expanding suite of publications. Identification involves obtaining the information from all relevant publications and comparing RFLP profiles from isolates of unknown identity with those that have previously been produced. Due to the large number of comparisons that must be made, this procedure is becoming increasingly cumbersome and difficult to achieve manually.

The time and effort required to make RFLP-based identifications would be substantially reduced if all available information were collated in a single, organised body of data, and if a rapid technique were devised for comparing the numerous profiles. Computer technology presents an appropriate tool for achieving both these goals. The aim of this study was, therefore, to develop an electronic database and automated search algorithm based on PCR-RFLP profiles to facilitate the identification of *Armillaria* isolates.

COMPUTER SOFTWARE DESIGN

Specific requirements

In order to be effective, a computerised RFLP-based identification tool must meet a number of criteria. It has to:

- Be compatible with different Microsoft® Windows® operating systems.
- Enable the user to store, change, extract and present data in the database.
- Compare RFLP data for an isolate entered by the user with those in the database.
- Take into account the fact that the user profile might not match any of the profiles in the database exactly; the closest match must, therefore, be returned as its probable identity.

Database design

The database was developed in Microsoft® Access. Data for the database were obtained from all previous publications containing RFLP profiles for *Armillaria* spp. (Tables 1 and 2). The design of the database and relationships among components and sub-components within the database are depicted in Fig. 1.

Application design

Code for this application was written in Microsoft® Visual Basic and has interactions with Microsoft® Access, Macromedia Flash and Microsoft® Word. Interaction between Microsoft® Visual Basic and Microsoft® Access takes place when data are being written to or extracted from the database. Macromedia Flash provides animation to the interface when the user is presented with options from menus within the application. Reports are generated through an interaction between Microsoft® Visual Basic and Microsoft® Word after data has been extracted from the database. The architecture of this application is depicted in Figs. 2 - 4.

Search algorithms for analyses

Algorithm 1 (Sum of differences - default): This algorithm calculate the summed squared deviation (S) between the user profile (I) and every profile in the database (D) that has the same number of fragments as the user profile. The summed deviation is calculated by squaring the difference between each fragment length in the user profile and the corresponding fragment length in the profile with which is being compared, and then taking the square root of the sum of these squared differences. Hence, the summed squared deviation between the user profile and profile i in the database is given by:

$$S_i = \sqrt{\sum_{j=1}^{n_i} (I_j - D_{ij})^2} \qquad (1)$$

where I_j is the length of Fragment j in the user profile, D_{ij} is the length of the corresponding fragment for Profile i in the database, and n_i is the number of fragments in each profile.

The database profile that yields the smallest value for S_i is then returned as the best match for the user profile.

Example: User profile = 350, 172 and 125 bp. (base pairs).

		Fragment number (j)			
		1	**2**	**3**	S_i
	D_1	350	180	119	$[(350\text{-}350)^2 + (172\text{-}180)^2 + (125\text{-}119)^2]^{1/2} = 10$
☺	D_2	348	175	120	$[(350\text{-}348)^2 + (172\text{-}175)^2 + (125\text{-}120)^2]^{1/2} = 6.16$
	D_3	345	172	130	$[(350\text{-}345)^2 + (172\text{-}170)^2 + (125\text{-}130)^2]^{1/2} = 7.07$
	I	*350*	*172*	*125*	

Thus, database profile D_2 ($S_2 = 6.16$) is the best much for the user profile.

Algorithm 2 (Normal distribution error): Algorithm 1, which was described above, only draws comparisons with those profiles in the database that have the same number of RFLP fragments as the user profile. If two similar fragment lengths are mistakenly entered as a single fragment in the user profile, Algorithm 1 would compare this profile with the wrong subset of profiles in the database, yielding incorrect results. A second algorithm was, therefore, developed. This algorithm rounds user and database RFLP fragment sizes to the nearest 5 bp. Fragment sizes are then distributed over a probability matrix with increments of 5 bp. S_i (Eqn 1) is then calculated, with the smallest value being the closest match.

Example: User profile = 454, 448 and 254 bp.

Initial dataset

		Fragment number (*j*)		
		1	**2**	**3**
(*i*)	D_1	448	249	
	D_2	451	302	247
	D_3	299	248	
	I	*454*	*448*	*254*

Is then converted to

		Fragment number (*j*)		
		1	**2**	**3**
(*i*)	D_1	450	250	
	D_2	450	300	245
	D_3	300	250	
	I	*455*	*450*	*255*

Probability matrix

	Fragmenth lengths																			S_i
	...	460	455	450	445	440	...	310	305	300	295	290	...	260	255	250	245	240	...	
D_1	0	0	0.5	1	0.5	0	...	0	0	0	0	0	...	0	0.5	1	0.5	0	...	1
D_2	0	0	0.5	1	0.5	0	...	0	0.5	1	0.5	0	...	0	0.5	1	0.5	0	...	1.58
D_3	0	0	0	0	0	0	...	0	0.5	1	0.5	0	...	0	0.5	1	0.5	0	...	2
I	0	0	0.5	1	0.5	0	...	0	0	0	0	0	...	0.5	1	0.5	0	0	...	

Thus, database profile D_1 ($S_1 = 1$) in the above example is the best match.

DISCUSSION

In this study we have developed an electronic database and search algorithm for rapid identification of *Armillaria* spp. using previously published RFLP profiles. This application allows the user to search, add, and update data in the database. Identification of unknown isolates using this application is achieved through search algorithms comparing user and database profiles to determine the closest match.

The computer program presented in this study is currently a stand-alone application for Microsoft® Windows®. All classes created in Microsoft® Visual Basic and animations developed in Macromedia Flash can be converted to function in a web-based environment. A future aim is thus to convert the application to function in a web-environment and to place it on a server at the Campus of the University of Pretoria (RSA) for use and update through the World Wide Web.

The application "RFLP Identification Tool for *Armillaria* species" was developed for identification of *Armillaria* species based only on ITS and IGS-1 PCR RFLP data. This application will, however, in future be expanded to incorporate RFLPs from other genes. It will also be made more informative regarding the species within the database by including information about species, descriptions, illustrations etc. At the present time, this application is restricted to identification and RFLP profiles pertaining to *Armillaria* spp., but it could be easily augmented in future to accommodate RFLP data for other genera of fungi.

LITERATURE CITED

Anderson JB, Stasovski E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycologia* **84**: 505 - 516.

Anderson JB, Ullrich RC. 1979. Biological species of *Armillaria mellea* in North America. *Mycologia* **71**: 402 - 414.

Banik MT, Volk TJ, Burdsall HH. 1996. *Armillaria* species of the Olympic Peninsula of Washington state, including confirmation of North America biological species XI. *Mycologia* **88**: 492 - 496.

Chillali M, Idder-Ighili H, Agustian A, Guillaumin J-J, Mohammed C, Botton B. 1997. Species delimitation in the African *Armillaria* complex by analysis of the ribosomal DNA spacers. *Journal of General and Applied Microbiology* **43**: 23 - 29.

Chillali M, Idder-Ighili H, Guillaumin J-J, Mohammed C, Lung Escarmant B, Botton B. 1998. Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European *Armillaria*. *Mycological Research* **102**: 533 - 540.

Coetzee MPA, Wingfield BD, Coutinho TA, Wingfield MJ. 2000a. Identification of the causal agent of Armillaria root rot of *Pinus* species in South Africa. Mycologia **92**: 777 - 785.

Coetzee MPA, Wingfield BD, Harrington TC, Dalevi D, Coutinho TA, Wingfield MJ. 2000b. Geographical diversity of *Armillaria mellea* s. s. based on phylogenetic analysis. *Mycologia* **92**: 105 - 113.

Guillaumin J-J, Berthelay S. 1981. Détermination spécifique des armillaires par la méthode des groupes de compatibilité sexuelle. Spécialisation écologique des espéces françaises. *Agronomie* **1**: 897 - 908.

Harrington TC, Wingfield BD. 1995. A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**: 280 - 288.

Hood IA, Redfern DB, Kile GA. 1991. *Armillaria* in planted hosts. In: Shaw CG, GA Kile, eds. *Armillaria Root Disease*. USDA Agricultural Handbook No. 691. Washington DC, USA: United States Department of Agriculture, 122 - 149.

Kile GA, McDonald GI, Byler JW. 1991. Ecology and disease in natural forests. In: Shaw CG, Kile GA, eds. *Armillaria Root Disease*. USDA Agricultural Handbook No. 691. Washington DC, USA: United States Department of Agriculture, 102 - 121.

Kim M-S, Klopfenstein NB, McDonald GI, Arumuganathan K, Vidaver AK. 2000. Characterization of North American *Armillaria* species by nuclear DNA content and RFLP analysis. *Mycologia* **92**: 874 - 883.

Kim M-S, Klopfenstein NB, McDonald GI, Arumuganathan K, Vidaver AK. 2001. Use of flow cytometry, fluorescence microscopy, and PCR-based techniques to assess intraspecific and interspecific matings of *Armillaria* species. *Mycological Research* **105**: 153 - 163.

Korhonen K. 1978. Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* **18**: 31 - 42.

Mwenje E, Wingfield BD, Coetzee MPA, Wingfield MJ. 2003. Molecular characterisation of *Armillaria* species from Zimbabwe. *Mycological Research* **107**: 291 - 296.

Otieno W, Pérez Sierra A, Termorshuizen A. 2003. Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya. *Mycologia* **95**: 160 - 175.

Pérez Sierra A, Whitehead DS, Whitehead MP. 1999. Investigation of a PCR-based method for the routine identification of British *Armillaria* species. *Mycological Research* **103**: 1631 - 1636.

Terashima K, Kawashima Y, Cha JY, Miura K. 1998. Identification of *Armillaria* species from Hokkaido by analysis of the intergenic spacer (IGS) region of ribosomal DNA using PCR-RFLP. *Mycoscience* **39**: 179 - 183.

Volk TJ, Burdsall HH, Banik MT. 1996. *Armillaria nabsnona*, a new species from western North America. *Mycologia* **88**: 484 - 491.

White EE, Dubetz CP, Cruickshank MG, Morrison DJ. 1998. DNA diagnostic for *Armillaria* species in British Columbia: within and between species variation in the IGS-1 and IGS-2 regions. *Mycologia* **90**: 125 - 131.

TABLE 1: Published IGS-1 PCR-RFLP profiles (published RFLP fragment size ranges are indicated in brackets but are not included in the database).

Species	Reference	RFLP Profile (bp)
IGS-1 digested with *Alu*I		
Armillaria sp.	Otieno *et al.* (2003)	310, 220, 135
A. borealis	Peréz Sierra *et al.* (1999)	305, 200, 100
A. borealis	"	305, 200, 135
A. borealis	Harrington and Wingfield (1995)	310, 200, 104
A. borealis	"	310, 200, 135
A. calvescens	"	582, 240
A. calvescens	Kim *et al.* (2000)	401 (6), 239 (4), 184 (2)
A. cepistipes	Peréz Sierra *et al.* (1999)	305, 200, 135
A. cepistipes	Kim *et al.* (2001)	309, 199, 137
A. cepistipes	Harrington and Wingfield (1995)	310, 200, 135
A. cepistipes	"	399, 200, 183
A. cepestipes	Peréz Sierra *et al.* (1999)	400, 200, 190
A. fuscipes	Mwenje *et al.* (2003)	380, 255, 130
*A. fuscipes**	Otieno *et al.* (2003)	380, 245, 135
A. fuscipes	Coetzee *et al.* (2000a)	365, 245, 135
A. gallica	Terashima *et al.* (1998)	317, 209, 135
A. gallica	Peréz Sierra *et al.* (1999)	390, 230, 190
A. gallica	Kim *et al.* (2000)	398 (2), 249 (5), 236 (2), 180 (3)
A. gallica	Harrington and Wingfield (1995)	399, 240, 183
A. gallica	Banik *et al.* (1996)	400, 235, 175
A. gallica	White *et al.* (1998)	400, 235, 190
A. gallica	Peréz Sierra *et al.* (1999)	400, 240, 190
A. gallica	White *et al.* (1998)	400, 245, 190
A. gallica	Peréz Sierra *et al.* (1999)	400, 250, 240, 190
A. gallica	Harrington and Wingfield (1995)	582, 240
A. gallica	Kim *et al.* (2000)	584 (8), 234 (4)
A. gallica	"	584 (8), 398 (2), 235 (3), 180 (2)
A. gemina	"	308 (3), 196 (2), 138 (1), 93 (3)
A. gemina	"	308 (3), 196 (2), 168 (2), 138(1), 93 (3)

TABLE 1 (continued)

Species	Reference	RFLP Profile (bp)
A. gemina	Harrington and Wingfield (1995)	310, 200, 135
A. heimii	Mwenje *et al.* (2003)	480, 255, 175
A. heimii	"	480, 230, 175
A. heimii	Coetzee *et al.* (2000a)	520, 220, 175
A. jezoensis	Terashima *et al.* (1998)	312, 250, 185
A. jezoensis	"	413, 308, 249, 185
A. jezoensis	"	417, 252, 187
A. mellea	Otieno *et al.* (2003)	310, 170
A. mellea	Peréz Sierra *et al.* (1999)	320, 155
A. mellea	Harrington and Wingfield (1995)	320, 155
A. mellea	Peréz Sierra *et al.* (1999)	320, 180, 155
A. mellea	Kim *et al.* (2000)	472 (6), 186 (2), 175 (1), 153 (1)
A. mellea	"	473 (7), 175 (2)
A. mellea	Harrington and Wingfield (1995)	490, 180
A. mellea subsp. *nipponica*	Terashima *et al.* (1998)	371, 162
A. nabsnona	Volk *et al.* (1996)	306 (299-314), 230 (223-237), 196 (191-202)
A. nabsnona	Kim *et al.* (2000)	308 (4), 229 (3), 196 (2)
A. nabsnona	White *et al.* (1998)	310, 225, 200
A. nabsnona	Harrington and Wingfield (1995)	534, 200
A. nabsnona	White et al. (1998)	535, 200
A. nabsnona	Kim *et al.* (2000)	541 (7), 197 (1)
A. nabsnona	"	541 (7), 308 (4), 229 (3), 196 (2)
A. nabsnona	Banik *et al.* (1996)	553 (490-615), 210
A. nabsnona	"	556 (513-598), 314 (302-327), 233 (221-246), 203(191-216)
A. nabsnona	Volk *et al.* (1996)	560 (541-581), 321 (311-332), 237 (229-245), 203 (197-210)
A. nabsnona	"	563 (552-575), 200 (144-206)
A. ostoyae	Peréz Sierra *et al.* (1999)	305, 200, 135
A. ostoyae	Kim *et al.* (2000)	308 (3), 196 (2), 138 (1)

TABLE 1 (continued)

Species	Reference	RFLP Profile (bp)
A. ostoyae	Kim *et al.* (2000)	308 (3), 196 (2), 138 (1), 93 (3)
A. ostoyae	Harrington and Wingfield (1995)	310, 200, 135
A. ostoyae	White *et al.* (1998)	310, 200, 135
A. ostoyae	Terashima *et al.* (1998)	312, 210, 137
A. ostoyae	Banik *et al.* (1996)	314 (309-319), 207 (203-211), 141(137-145)
A. sinapina	Harrington and Wingfield (1995)	399, 200, 135
A. sinapina	Kim *et al.* (2001)	401, 241, 186
A. sinapina	White *et al.* (1998)	400, 200, 135
A. sinapina	"	400, 200, 190
A. sinapina	"	400, 200, 190, 135
A. sinapina	"	400, 235, 190
A. sinapina	"	400, 235, 200, 190, 135
A. sinapina	Kim *et al.* (2000)	401 (4), 239 (4), 196 (2), 184 (2), 139 (1)
A. sinapina	"	401 (6), 239 (4), 184 (2)
A. sinapina	Banik *et al.* (1996)	401 (391-410), 237 (299-245), 184 (177-191)
A. sinapina	Kim *et al.* (2000)	402 (7), 196 (2), 184 (2), 139 (1)
A. sinapina	Terashima *et al.* (1998)	423, 258, 190
A. singular	"	410, 207, 184
A. singular	"	417, 266, 186
A. tabescens	Harrington and Wingfield (1995)	320, 240, 100
A. tabescens	Peréz Sierra *et al.* (1999)	430, 240
A. tabescens	Harrington and Wingfield (1995)	430, 240
NABS X	"	399, 183, 142
NABS X	Kim *et al.* (2001)	401, 186, 144
NABS X	"	401 (3), 184 (1), 145 (1)
NABS XI	"	401 (3), 197 (1), 184 (1)
NABS XI	"	401, 197, 186
NABS XI	Banik *et al.* (1996)	413 (389-436), 203 (198-207), 185

TABLE 1 (continued)

Species	Reference	RFLP Profile (bp)
IGS-1 digested with *Dde* I		
A. gallica	Terashima *et al.* (1998)	237, 211, 148
A. jezoensis	"	235, 222, 147, 112
A. ostoyae	"	214, 179, 120
A. sinapina	"	235, 218, 148, 111
A. singular	"	234, 150, 113
IGS-1 digested with *Bsm*I		
A. ostoyae	Peréz Sierra *et al.* (1999)	600, 300
A. ostoyae	Harrington and Wingfield (1995)	620, 300
IGS-1 digested with *Nde*I		
A. borealis	Harrington and Wingfield (1995)	550, 370
A. borealis	Peréz Sierra *et al.* (1999)	565, 380
A. gemina	Kim *et al.* (2000)	913, 552, 461, 372
A. ostoyae	Harrington and Wingfield (1995)	550, 370
A. ostoyae	Peréz Sierra *et al.* (1999)	565, 380
A. ostoyae	Kim *et al.* (2000)	552, 372
IGS-1 digested with *Hind*II		
A. cepistipes	Harrington and Wingfield (1995)	580, 340

* As *A. heimii*

TABLE 2: Published ITS PCR-RFLP profiles.

Species	Reference	RFLP Profile (bp)
ITS digested with *Alu*I		
*A. fuscipes**	Otieno *et al.* (2003)	480, 160, 85
A. heimii	Chillali *et al.* (1997)	530, 72
A. heimii	"	530, 72
A. mellea	Otieno *et al.* (2003)	320, 235, 190, 150
A. mellea subsp *africana*	Chillali *et al.* (1997)	390, 271, 150, 72
Armillaria SIG III	Chillali *et al.* (1997)	540, 234, 72
Armillaria sp.	Otieno *et al.* (2003)	510, 225, 95
ITS digested with *Cfo* I		
A. borealis	Chillali *et al.* (1998)	400, 350, 92
A. cepistipes	"	400, 350
A. ectypa	"	500, 350
A. gallica	"	400, 350
A. ostoyae	"	400, 350
A. tabescens	"	500, 350
ITS digested with *EcoR* I		
A. borealis	Chillali *et al.* (1998)	510, 330
A. cepistipes	"	510, 330
A. ectypa	"	500, 330
A. gallica	"	510, 330
A. heimii	Chillali *et al.* (1997)	315
A. heimii	"	315
A. mellea subsp *africana*	"	500, 360
A. ostoyae	Chillali *et al.* (1998)	510, 330
A. tabescens	"	510, 330
Armillaria SIG III	Chillali *et al.* (1997)	500, 360

TABLE 2 (continued)

ITS digested with *Hin*fI		
A. borealis	Chillali *et al.* (1998)	310, 234, 170, 110
A. cepistipes	"	310, 234, 130, 110
*A. fuscipes**	Otieno *et al.* (2003)	220, 190, 170, 72
A. gallica	Chillali *et al.* (1998)	310, 234, 130, 110
A. gallica	"	310, 234, 118, 90
A. heimii	Chillali *et al.* (1997)	271, 234, 100
A. heimii	"	420, 234
A. mellea	Otieno *et al.* (2003)	280, 180, 170, 140, 100
A. mellea subsp. *africana*	Chillali *et al.* (1997)	400, 234, 200
A. ostoyae	Chillali *et al.* (1998)	310, 234, 170, 110
Armillaria SIG III	Chillali *et al.* (1997)	460, 281, 200
Armillaria sp.	Otieno *et al.* (2003)	360, 230, 150, 100
ITS digested with *Nde* II		
*A. fuscipes**	Otieno *et al.* (2003)	390, 250
A. heimii	Chillali *et al.* (1997)	369, 271
A. heimii	"	369, 271
A. mellea	Otieno et al. (2003)	280, 240, 230, 150
A. mellea subsp. *africana*	Chillali *et al.* (1997)	281, 234, 230, 141
Armillaria SIG III	"	603, 230
Armillaria sp.	Otieno *et al.* (2003)	590, 270

* as *A. heimii*

Figure 1. Design of the RFLP database. Black boxes are the components and open boxes the sub-components of each component. Numbers and M (many) indicate the relationship (1:1 or 1:M) between two components or between a component and a sub-component.

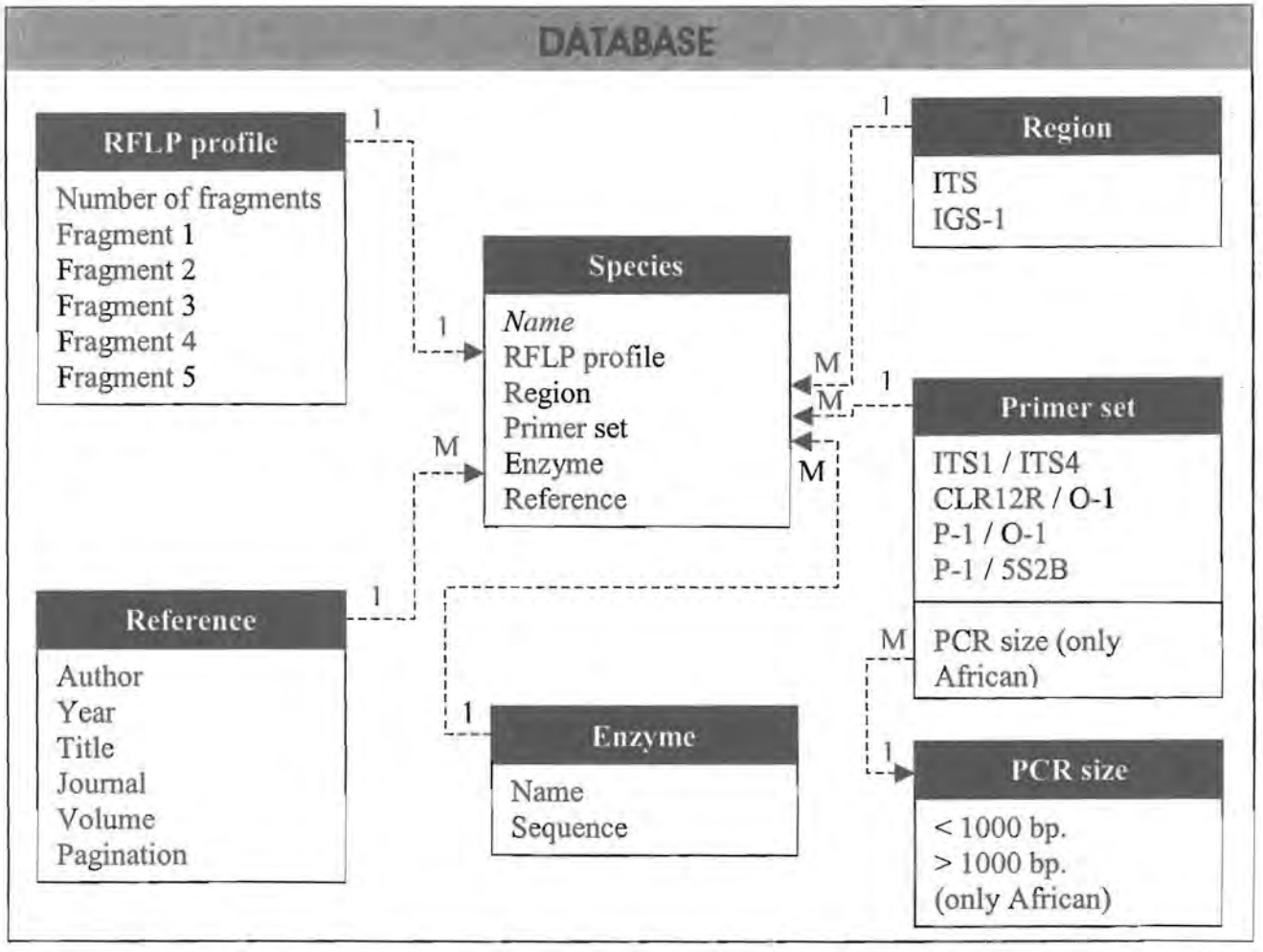

DATABASE
RFLP profile
Number of fragments
Fragment 1
Fragment 2
Fragment 3
Fragment 4
Fragment 5
Species
Name
RFLP profile
Region
Primer set
Enzyme
Reference
Region
ITS
IGS-1
Primer set
ITS1 / ITS4
CLR12R / O-1
P-1 / O-1
P-1 / 5S2B
PCR size (only African)
Reference
Author
Year
Title
Journal
Volume
Pagination
Enzyme
Name
Sequence
PCR size
< 1000 bp.
> 1000 bp.
(only African)
1
M

Figure 2. Architecture of the "RFLP Identification Tool for *Armillaria* species" computer application. Down arrows (⇳) indicate drop-down-menus with data from the database. Black boxes show the entities in the data base from Fig. 1.

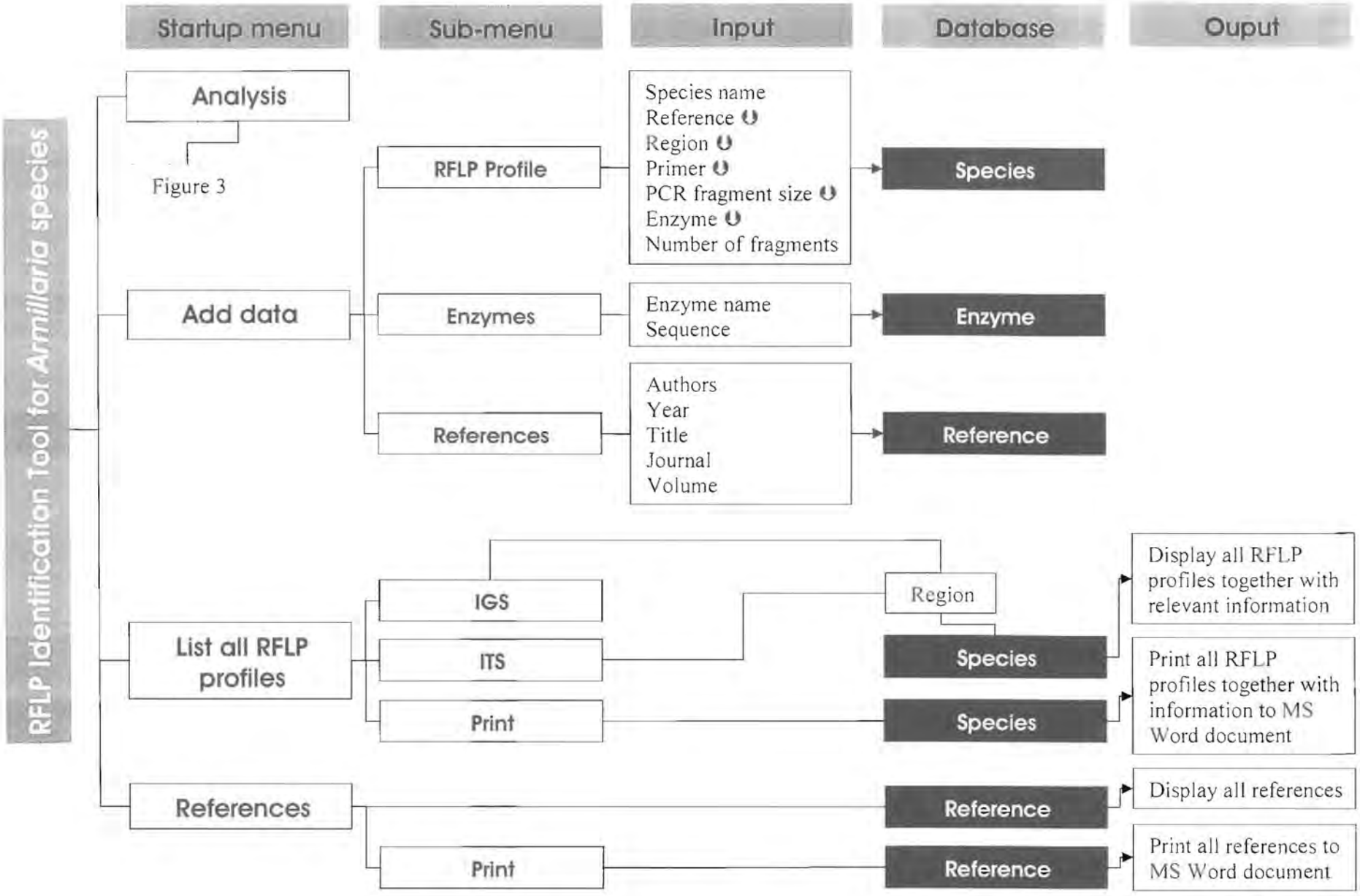


Figure 3. Analyses of user profiles. The user chooses one of the two algorithms to calculate the best much between the user-profile and the profiles selected from the procedure outlined in Fig. 4. The species name, RFLP profile and reference for the best as well as close matches are given as output.

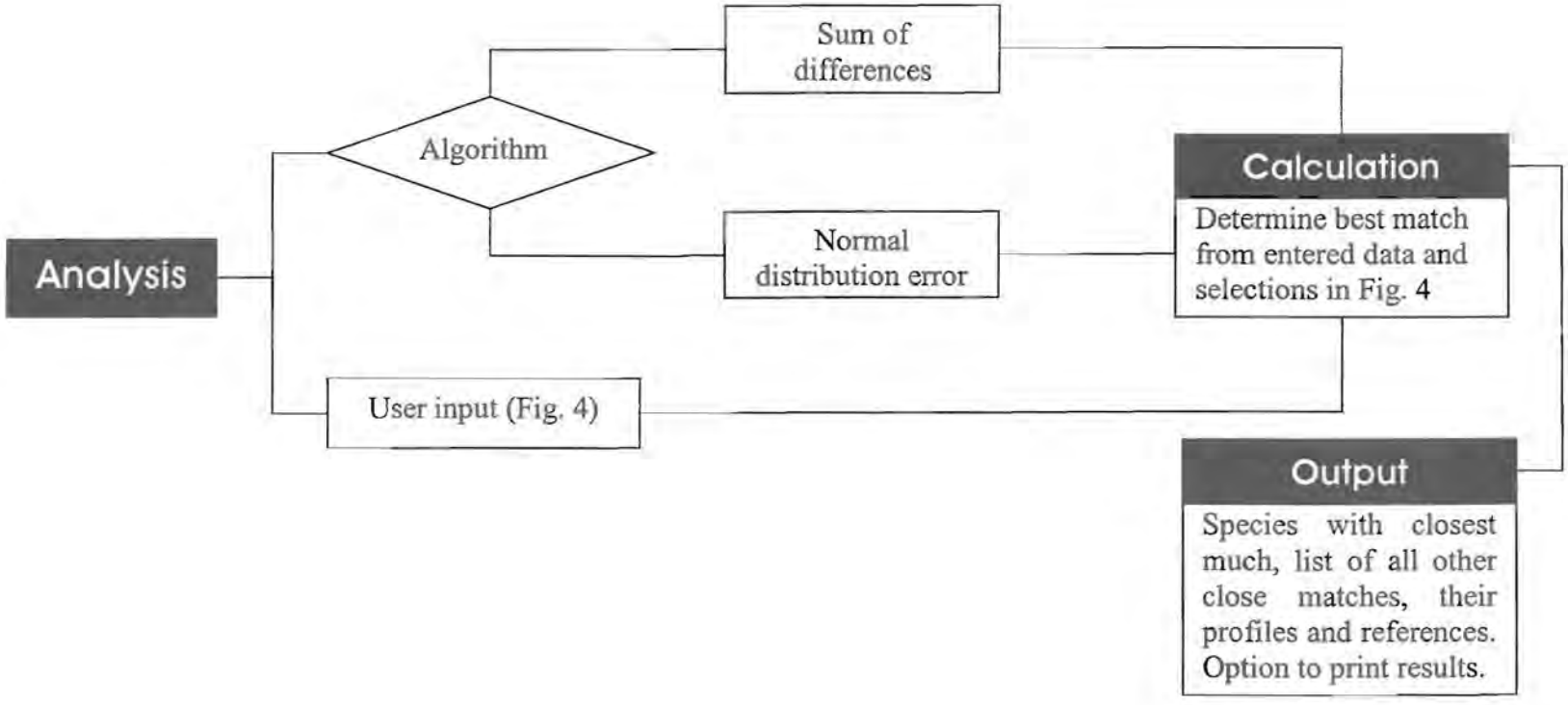
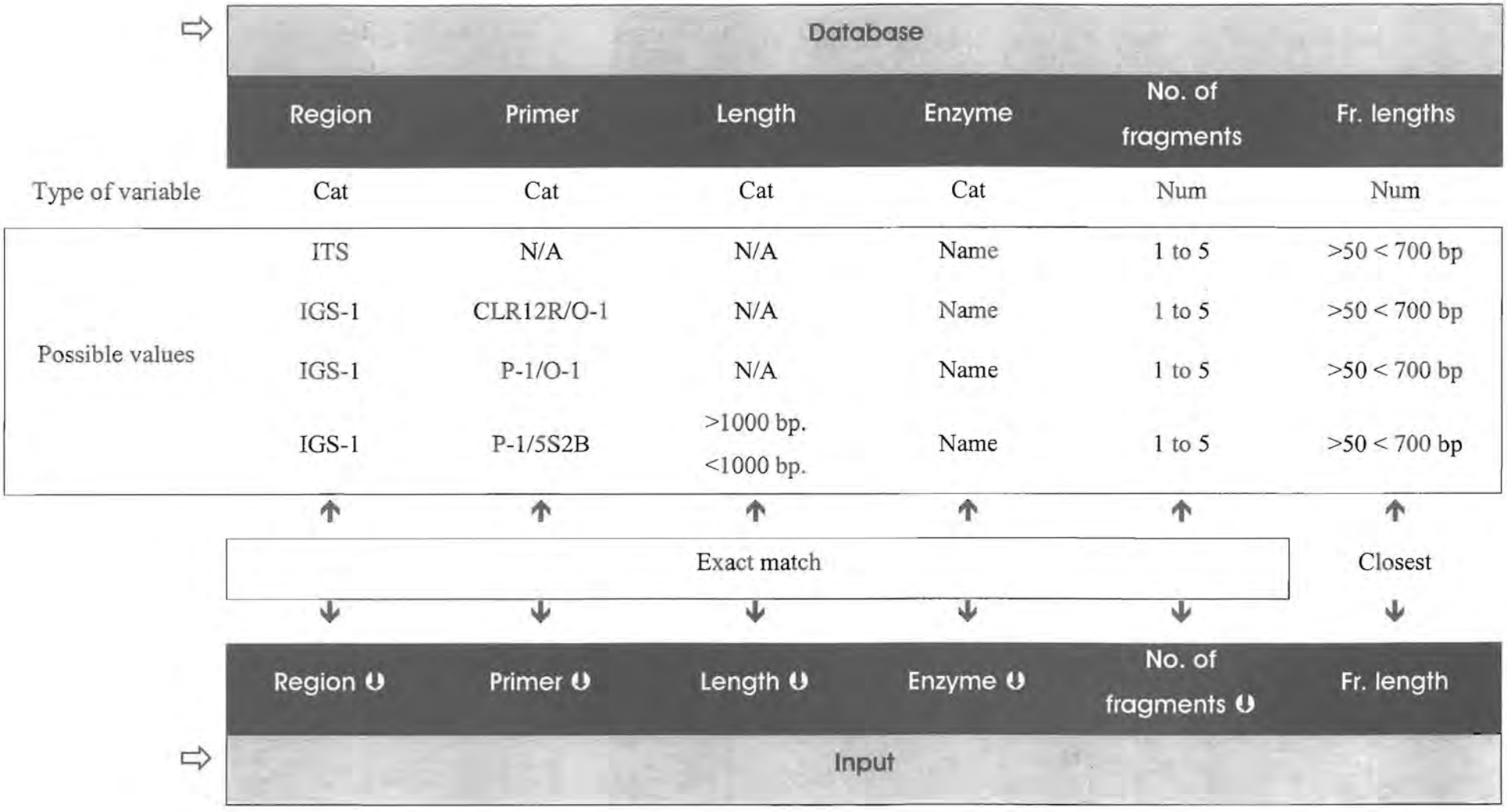


Figure 4. Interaction between database and application interface before calculating the best much between user and database profiles. The type of variables are either categorical (Cat), chosen via drop-down-menus or numerical (Num), provided by the user. Vertical arrows (⇨) indicate the directional sequence of events and encircled arrows (⓫), drop-down-menus with data from the database.



DESCRIPTION OF THE USER GRAPHICAL INTERFACE

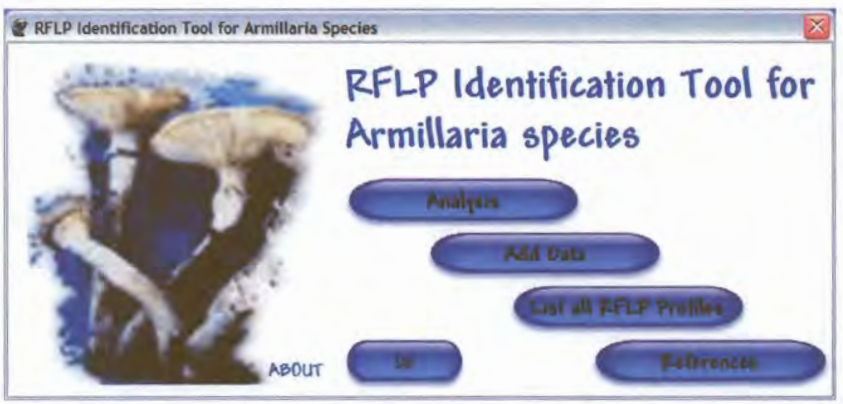
REQUIREMENTS:

System: Windows® 95, Windows® 98, Windows® 2000, Windows® XP

Additional: Microsoft® Word

Best viewed at: 1152 x 864 pixels, 32 bit colour

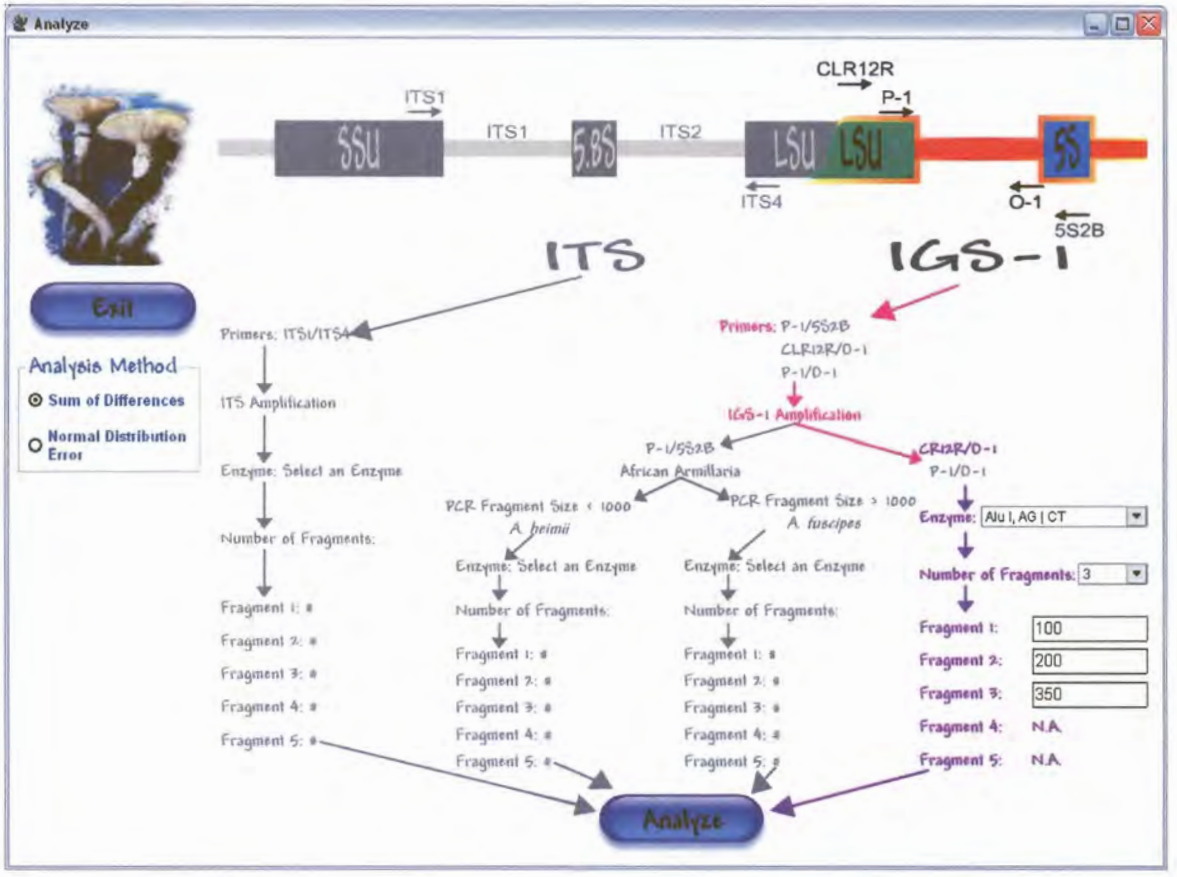
GENERAL MENU:



The General (start) menu gives the five options:

- *Analysis:* RFLP profile analysis function of the application.
- *Add data*: RFLP profiles, enzymes or references can be added, edited or removed from the database.
- *List all RFLP profiles*: IGS-1 or ITS RFLP profiles are listed from the database and can be printed to a Microsoft® Word file.
- *References*: Lists all references from the database and exports references to a Microsoft® Word file if required.
- *Exit:* Exits the programme.

ANALYSIS WINDOW

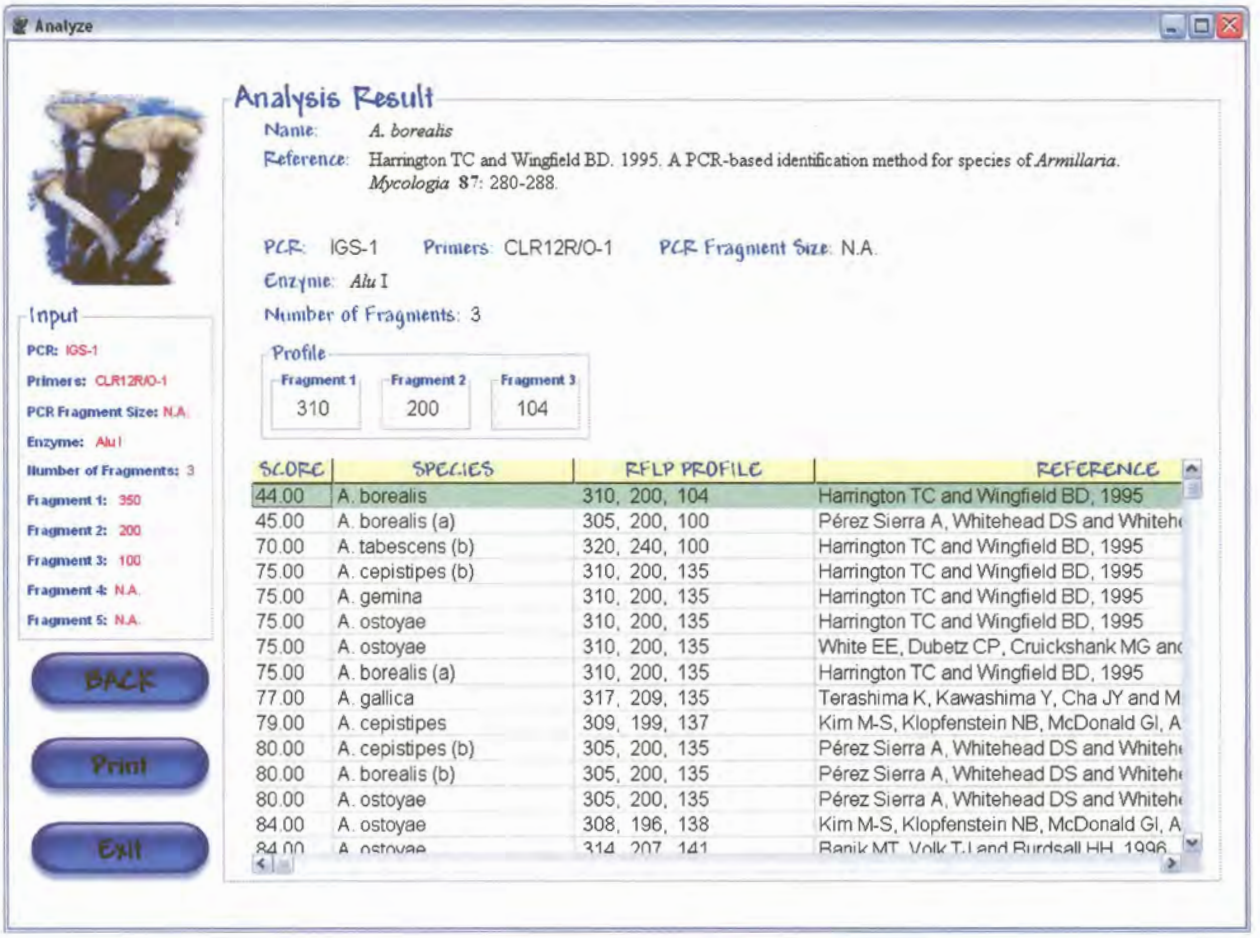


User RFLP profiles are analysed by following the steps outlined below:

1) ITS or IGS-1 rDNA region is selected by moving the mouse-pointer over the required region. The activated region change colours when selected.
2) A primer-pair is selected by moving the mouse-pointer over one of the primer-pairs; change in colour indicates the selected primer-pair.
3) Choosing primer pair P-1 / 5S2B will activate options for the PCR fragment size of the African *A. fuscipes* (> 1000 bp.) or *A. heimii* (< 1000 bp.).
4) An enzyme is selected from a drop-down menu that lists all enzymes and their restriction sequences from the database.
5) The number of fragments (between 1 and 5) to be entered are selected from a drop-down menu.
6) Boxes for the fragment sizes of the RFLP profile open after Step 4. Only fragment sizes between 50 and 700 bp. are accepted.
7) The Analyze button is pressed to execute the analysis.

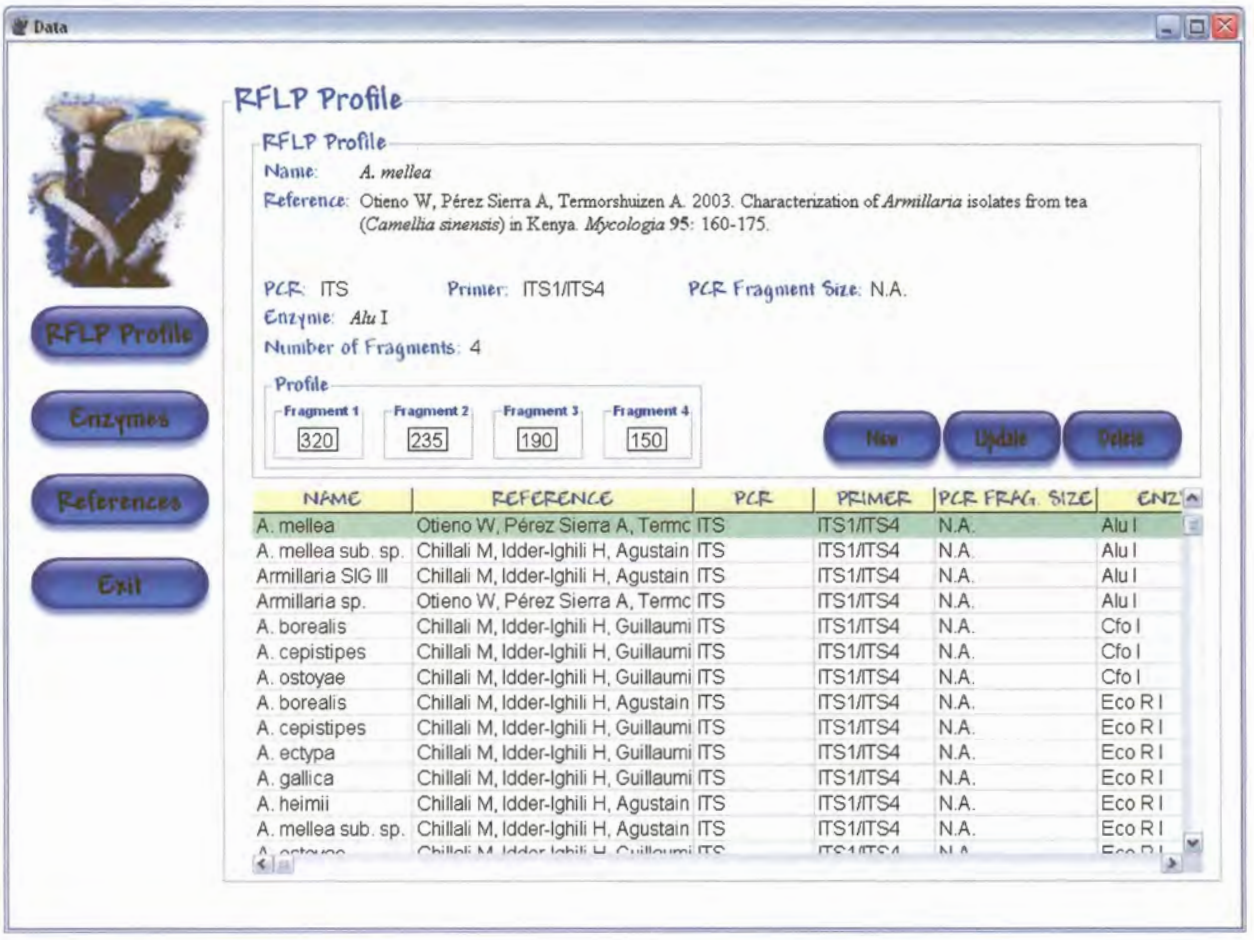
One of two analysis algorithms, sum of differences and normal distribution error, can be selected by the user for the analysis.

ANALYSIS RESULT WINDOW



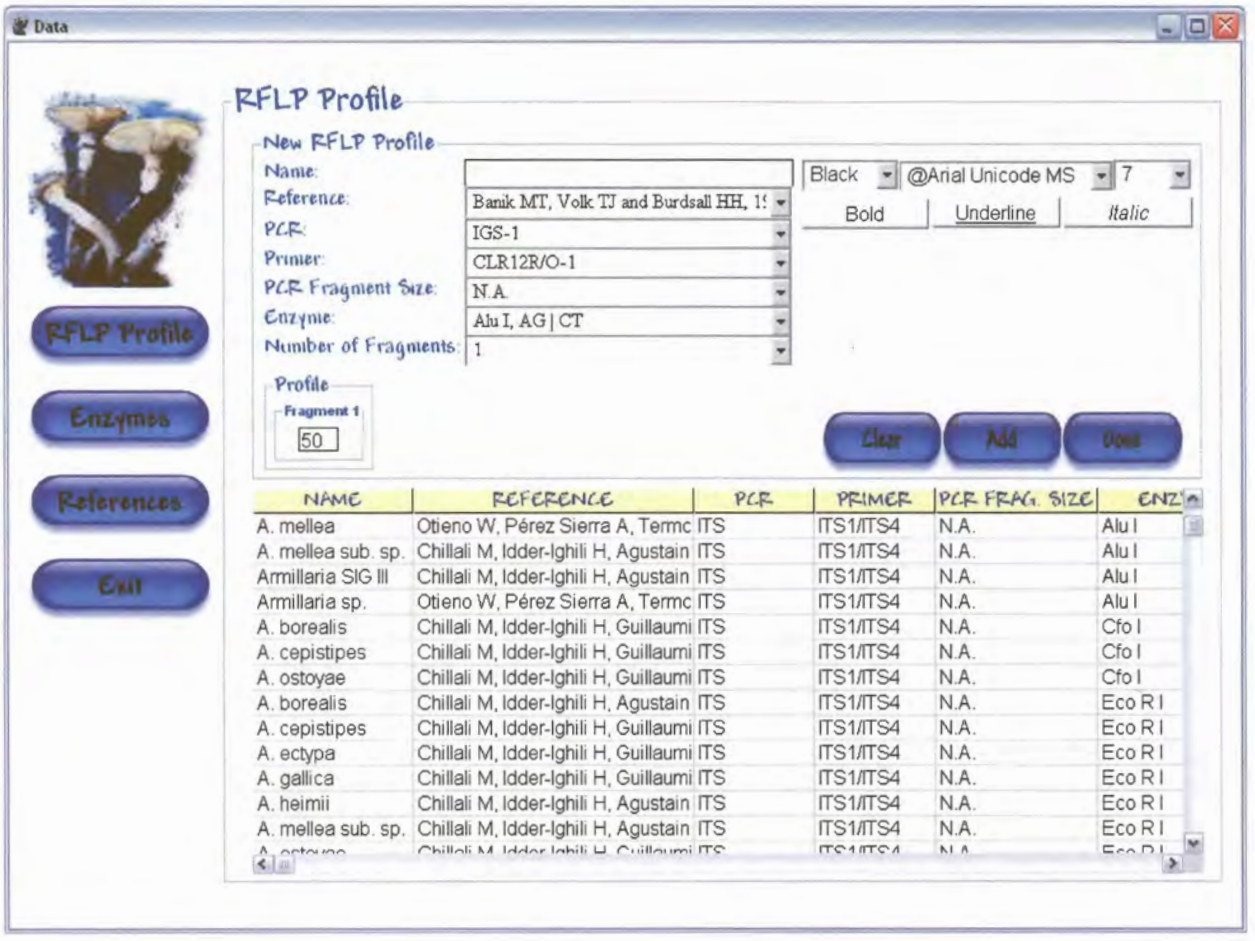
The Result window gives a list of species, in the order of the best to the worst match with the user RFLP profile, together with all their information. Detailed information pertaining to a specific species record (name, full reference, PCR region, primer pairs, PCR fragment sizes, enzyme used, number of fragments and fragment sizes) is displayed after selecting the record from the table. The table can also be exported to a Microsoft® Word file using the Print button. The Back button will take the user to the analysis window, which will still contain all the selected and entered data; these can be changed and the analysis repeated. The Exit button returns the user to the General window.

ADD DATA – RFLP PROFILE WINDOW



RFLP profile records can be added, updated or deleted within the Add Data window after pressing the RFLP Profile button. This procedure requires the following steps:

1) A list of all records within the database is displayed in a table.
2) A specific record can be selected with the mouse-pointer by clicking on any of the items in the table; this will highlight the selection and show detailed information regarding the species.
3) The selected record can then be updated or deleted using the Update and Delete buttons.

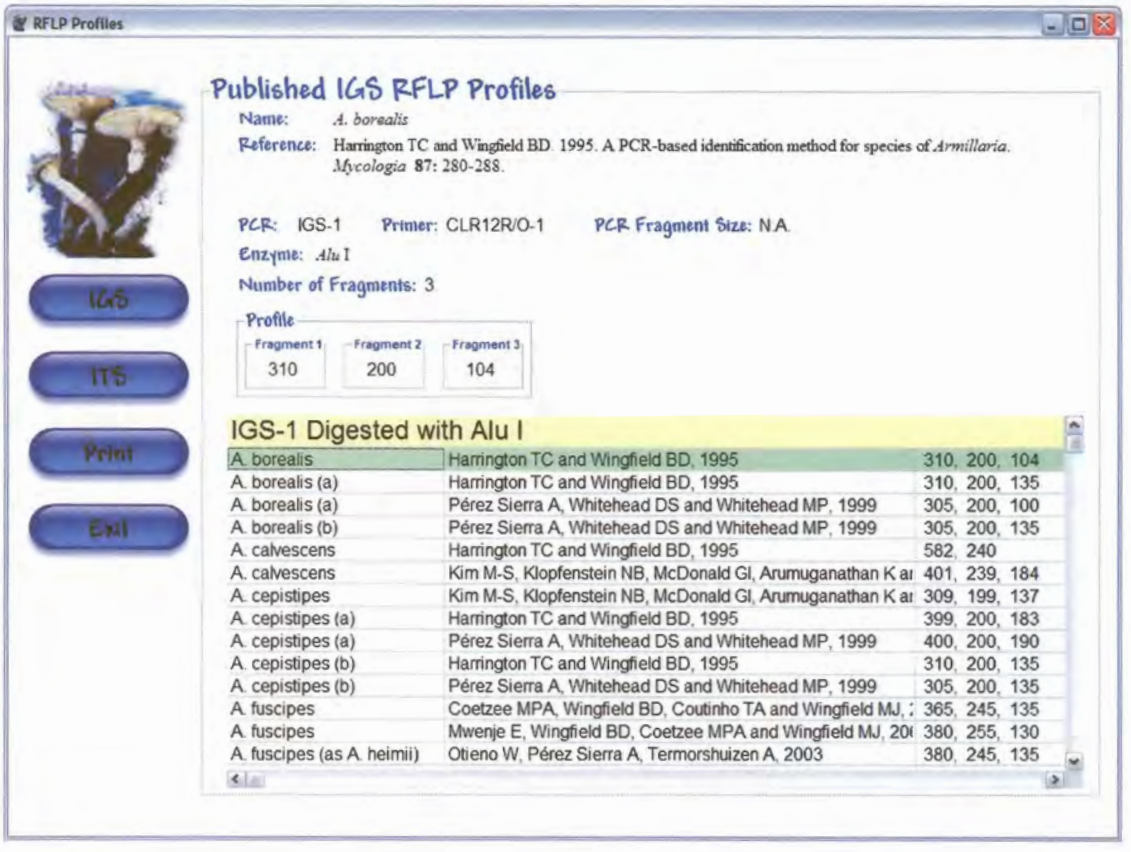


New RFLP profiles are added to the database by pressing the New button (see screen on previous page).

1) Data are entered by the user or selected from pull-down-menus.
2) The Add button will add the data to the database and the next record can then be entered.
3) The Done button will close the Add function and the new records are displayed in the table.

The procedure outlined above is also applicable to adding, updating and removing enzymes and reference records.

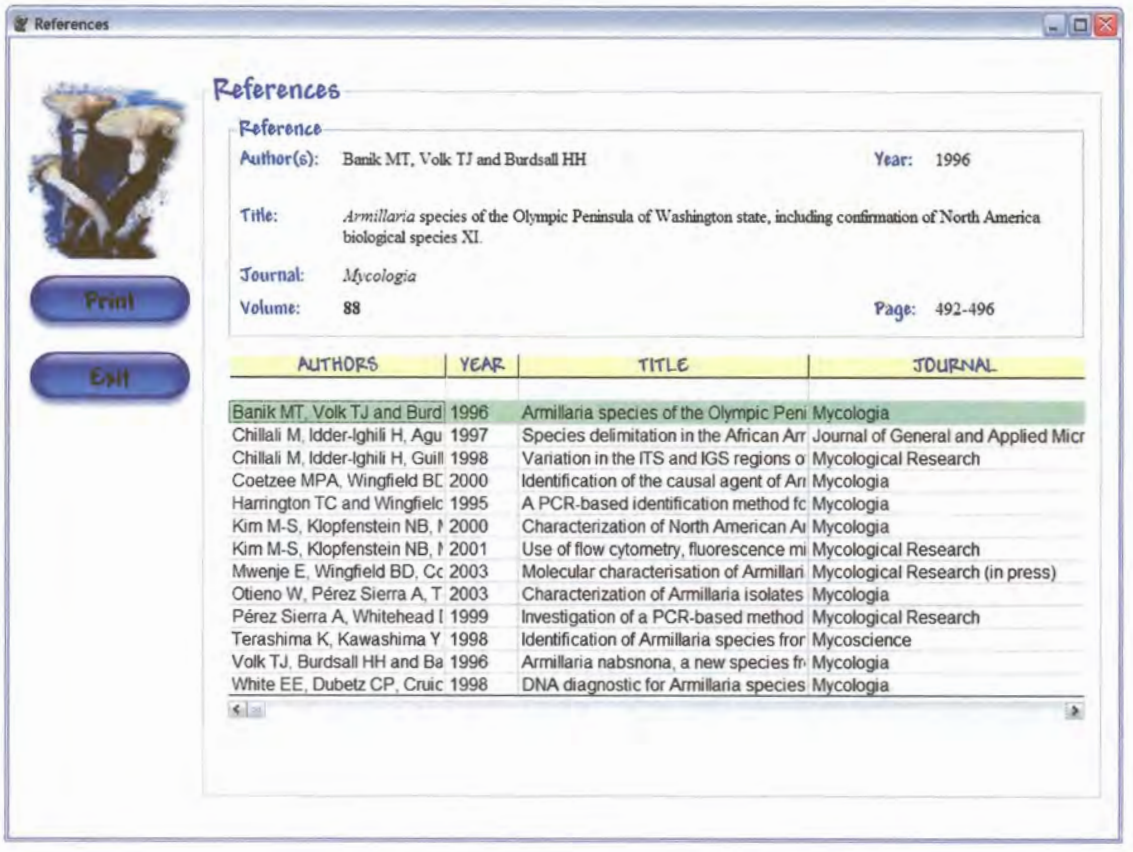
LIST ALL PROFILES WINDOW



Selected subsets of RFLP profiles can be displayed following the steps outlined below:

1) *An rDNA region is selected using the IGS or ITS buttons.*
2) A table with all records in the database pertaining to that region is displayed.
3) A specific record can be selected from the database in the table.
4) The Selected record is highlighted and detailed information is displayed.
5) A list of all the records in the database pertaining to the selected rDNA region can be exported to Microsoft® Word using the Print button.
6) The Exit button returns the user to the General window.

LIST ALL REFERENCES WINDOW



References stored in the database are viewed through the following steps:

1) A table with all records in the database is displayed.
2) A specific record can be selected from the database.
3) The selected record is highlighted and detailed information is displayed.
4) A list of all the reference records can be exported to Microsoft® Word using the Print button.
5) The Exit button returns the user to the General window.